2002

THE ROLE OF NF-kB ACTIVATION IN HEPATIC TUMOR PROMOTION BY POLYCHOLORINATED BIPHENYLS (PCBs)

Zijing Lu
University of Kentucky, zlu0@uky.edu

ABSTRACT OF DISSERTATION

Zijing Lu

The Graduate School

University of Kentucky

2002

THE ROLE OF NF-κB ACTIVATION IN HEPATIC TUMOR PROMOTION BY POLYCHOLORINATED BIPHENYLS (PCBs)

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in
The Graduate School
at the University of Kentucky

By

Zijing Lu

Lexington, Kentucky

Director: Dr. Howard Glauert, Professor, Graduate Center for Nutritional Sciences

Lexington, Kentucky

2002

ABSTRACT OF DISSERTATION

ABSTRACT OF DISSERTATION

THE ROLE OF NF-κB ACTIVATION IN HEPATIC TUMOR PROMOTION BY POLYCHOLORINATED BIPHENYLS (PCBs)

Polychlorinated biphenyls (PCBs) are nongenotoxic hepatic tumor promoters. PCBs have been shown to cause oxidative stress, but the exact mechanism by which PCBs exert their tumor promoting activity is not clear. In our study, PCB-153, a non-coplanar congener, caused a transient increase in hepatic NF-κB DNA binding activity and cell proliferation, while PCB-77, a coplanar congener, showed no effect. Our second study using a mouse model that was deficient in the p50 subunit of NF-κB (p50-/-) showed that NF-κB contributes to the changes in hepatocyte proliferation and apoptosis in response to PCB-153 treatment: a single dose of PCB-153 increased hepatic NF-κB activity and cell proliferation in wild type mice, but not in the p50-/- mice; longer-term treatment with PCB-153 increased cell proliferation in p50-/- mice, but this increase was less than that in the wild type. In addition, p50-/- livers had more apoptosis than in the wild type, and PCB-153 inhibited apoptosis in the p50-/- livers. p50-/- livers had less cyclin D1 protein than the wild type, but that the mRNA levels were same. Bcl-x_L protein was not changed by PCB-153, and wild type and p50-/- mice had the same level of Bcl-x_L protein. In the third study, PCB-77 caused an increase in hepatic NF-κB DNA binding

activity and cell proliferation during the promotion stage, and this increase was blocked by dietary supplementation of vitamin E, but the number and volume of placental glutathione *S*-transferase (PGST)-positive foci were slightly, though insignificantly, increased in the same animals. The apparent conflict could be due to different effect in different cells: high level vitamin E significantly inhibited PCB-77-induced cell proliferation in normal hepatocytes, while this inhibitory effect was much less in the PGST-positive hepatocytes. In conclusion, our studies show that a non-coplanar PCB can cause an increase in hepatic NF-κB DNA binding activity in rats and mice, and this increase contributes to the change in cell proliferation and apoptosis. Dietary vitamin E supplementation did not show protective effect on the formation of altered hepatic foci that were promoted by PCBs, although vitamin E supplementation decreased PCBs-induced hepatic NF-κB activation and cell proliferation.

KEYWORDS: Liver, NF-κB, Polychlorinated biphenyls (PCBs), tumor promotion, Vitamin E

THE ROLE OF NF-κB ACTIVATION IN HEPATIC TUMOR PROMOTION BY POLYCHOLORINATED BIPHENYLS (PCBs)

By

Zijing Lu

_____
Director of Dissertation

_____
Director of Graduate Studies

_____

RULES FOR THE USE OF DISSERTATIONS

Unpublished dissertations submitted for the Doctor's degree and deposited in the University of Kentucky Library are as a rule open for inspection, but are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but quotations or summaries of parts may be published only with the permission of the author, and with the usual scholarly acknowledgments.

Extensive copying or publication of the dissertation in whole or in part also requires the consent of the Dean of the Graduate School of the University of Kentucky.

A library that borrows this dissertation for use by its patrons is expected to secure the signature of each user.

Name	Date

DISSERTATION

Zijing Lu

The Graduate School

University of Kentucky

2002

THE ROLE OF NF-κB ACTIVATION IN HEPATIC TUMOR PROMOTION BY POLYCHOLORINATED BIPHENYLS (PCBs)

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in
The Graduate School
at the University of Kentucky

By

Zijing Lu

Lexington, Kentucky

Director: Dr. Howard Glauert, Professor, Graduate Center for Nutritional Sciences

Lexington, Kentucky

2002

ACKNOWLEDGMENTS

There are multiple people whom I would like to thank toward the completion of this dissertation. Foremost, I would like to thank my advisor, Dr. Howard Glauert. Dr. Glauert, your guidance and input toward my graduate studies have been immeasurable. I would also like to thank my committee members, Drs. Brett Spear, Larry Robertson, Ching K. Chow, and Zhigang Wang for their input on my project as well as my outside member, Dr. Daniel J. Noonan.

I have received tremendous assistance from colleagues who I have been working with during my study at University of Kentucky. In particular, I want to mention Job, Karen, Michelle, Petruta, and Casey, Dr. Robertson's laboratory, and Dr. Spear's laboratory.

I would not be pursuing my doctorate without the continued love and support from my parents and my brothers and sister. I thank them immensely for helping me become what I am today.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1. Introduction

l. Introduction to Polychlorinated Biphenyls

A. Background:

Polychlorinated biphenyls (PCBs) are a family of chemicals sharing a basic shape: a biphenyl ring structure with one to ten chlorine atoms placed in any of ten open positions on the ring structure. Therefore, there are 209 possible congeners or isomers of PCBs (Safe 1994).

Most PCB congeners are colorless, odorless crystals. The commercial mixtures are clear viscous liquids. PCBs have low water solubilities and low vapor pressures, while they are soluble in most organic solvents, oils, and fats. PCBs usually are very stable and do not degrade easily, and they can be destroyed only under certain conditions by chemical, thermal, and biochemical processes. Usually the processing of PCB waste requires high temperatures.

B. Production of Polychlorinated Biphenyls:

PCBs were first synthesized by Schmidt and Schulz in 1881, but industrial production was not started until 1920's. The industrial production of PCBs consisted of multiple congener mixtures and was sold under different brand names. In the United States, PCBs were produced by the Monsanto Chemical Company under the name "Aroclor", and had individual production groups labeled as 1221, 1232, 1242, 1248, 1254 and 1260, in which the last two digits represent the percentage of chlorine by weight in the mixture. PCBs were also produced and sold under different trade name in other countries, including Germany (Bayer, Clophens), France (Prodelec, Phenoclors), Italy (Caffro, Fenoclors), Japan (Kanegafuchi, Kanechlors), Russia (Sovol, Sovol), Poland (Zabkowice Slaskie, Chlorofen), Spain (Cross, Fenochlors) and Czechoslovakia (Chemko, Delor). Their production was banned in North America and Western Europe in the 1970's, and in Eastern Europe and Russia in the 1990's. The overall production of PCBs mixtures worldwide has been determined as 1.2 million metric tons, and the United States accounted for 35% of the world distribution of PCBs (Safe 1994).

PCBs have been used extensively in many industrial settings because of their stability under a broad range of chemical, thermal and electrical conditions. They were used in transformers and capacitors, in the formulation of lubricating and cutting oils, in pesticides and flame retardants, and as plasticizers in paints, copying paper, adhesive, sealants and plastics.

C. Environment Pollution:

Due to their physical properties, PCBs have been widely used since the 1920's, but until the development of sensitive detection system their contamination in environment was not understood. PCBs were first detected as an environmental contaminant by a gas chromatography method in 1966 (Jensen *et al*. 1969). After that, studies have shown that PCBs are present in the environment worldwide in varying concentrations, but mostly are found at the pg/m^3 level in the atmosphere, ng/kg in surface water, and μg/kg in sediment and soils (Booij & van Drooge 2001; Fromberg *et al*. 1999; Hansen 2000; Rosinska & Sulkowski 2001).

Because of their lipophilicity and stability, PCBs bioconcentrate and bioaccumulate in the food chain, which has been demonstrated by the observation that high levels of PCBs concentration were found in fish fat and eggs of aquatic birds (in the mg/kg range) (Giesy *et al*. 1995; Giesy *et al*. 1994).

D. PCBs exposure:

Humans are exposed to PCBs through inhalation of airborne particles, occupational exposure, and ingestion of food containing PCBs. Workers employed at transformer and capacitor manufacturing plants are among those most often exposed to PCBs through their occupation. The general public is exposed to PCBs from inhalation of particulates, or ingestion of contaminated food. The sources of food contamination include food packaging, crops grown on contaminated soil, contaminated feed for animals, or seafood from environmentally contaminated regions. The increased level of PCBs have been found in human serum, blood, adipose tissue, and human milk and milk fat; all these may due to the different routes of human exposure to PCBs (Safe 1994; Silkworth & Brown 1996). In addition, there are two significant PCB poisoning of

humans, the Yusho and Yucheng incidents, which occurred in Japan and Taiwan respectively.

Yusho and Yucheng incidents:

In 1968, a mass food poisoning occurred in western Japan, involving more than 1,850 people. The poisoning is now understood to have been caused by ingestion of a commercial brand of rice oil (Yusho) contaminated with polychlorinated derivatives of biphenyls, dibenzofurans, quaterphenyls, and some other related compounds. This poisoning caused acute toxicity including chlorance and skin pigmentation alteration, irritation of the eyes as well as increased discharge, fever, jaundice, headache, numbness in the limbs, general fatigue and weakness, as well as liver dysfunction (Okumura 1984). Epidemiological studies following this population showed offspring having darkly pigmented skin that faded by the age of five months (Yamashita & Hayashi 1985), and adults having respiratory distress, with major chlorance subsiding after about fifteen years (Nakanishi *et al*. 1985). An increased incidence of hepatocellular carcinoma has been linked to the PCB exposure in this population (Kikuchi 1984).

In 1979, an outbreak of food poisoning occurred in Taiwan, involving more than 2,000 people. The event was also caused by ingestion of PCBs mixtures-contaminated rice oil (Yucheng). The symptoms observed in this population were similar to those seen in the Yusho incident, including skin diseases, liver damage, immunosuppression, and neuropathy (Ikeda 1996). The serum levels of PCBs in women of this population were still above average after 14 years (Guo *et al*. 1997). Chronic toxicity that has been attributed to PCB in this population includes reproductive dysfunction, liver and lung cancer (Guo *et al*. 1999; Ikeda 1996; Yu *et al*. 1997).

Occupational exposure:

Studies have examined individuals who were exposed to PCBs during their employment years, mainly in those who ever worked in capacity and transformer plants. Studies on the adverse effects of PCBs on groups of occupational exposed workers have shown effects such as chloracne and dermal lesions, decrease in pulmonary function, decrease in body weight in children from exposed mother, eye irritation, and liver

dysfunction including hepatomegaly, increased liver enzymes, and induction of drug-metabolizing enzymes (Safe 1994). Generally no increased mortality was ever found in those workers, although there was a link between PCB exposure and malignant melanoma and cancer of the brain (Sinks *et al*. 1992), and between PCBs exposure and pancreatic cancer (Yassi *et al*. 1994).

General public exposure:

Due to their non-polar, lipophilic physical properties and their resistance to biochemical degradation, PCBs tend to accumulate in fatty tissues in humans. PCB levels in human have been studied for over 30 years. The general conclusions of PCBs in human are that PCBs are ubiquitous, with average levels in adipose around ppm level (Aronson *et al*. 2000; Hansen 2000; Safe 1994), and in blood around ppb levels (Hansen 2000; Hunter *et al*. 1997). Not all humans have the same level of PCB in their body, because of different exposure levels from food, occupation, environment, and other sources.

2. PCBs and cancer

A. Summary of studies on PCBs and specific types of cancer

Although studies showed no increase in overall mortality rate in the PCBs-exposed groups, accumulating data have shown the association of PCBs exposure and specific types of tumors in human and experimental animals.

Epidemiological studies

PCBs and brain cancer

There was only one study describing the possible association of cancer of the brain and nervous system and PCBs exposure (Sinks *et al*. 1992). Although the brain cancer mortality was 2.5 fold higher than expected in the study group of electrical capacitor manufacturing workers, the possibility that the increased incidence of brain cancer is due to chance, bias, and confounding factor cannot be excluded in this study.

PCBs and breast cancer

The possible role of PCBs in breast cancer development has drawn much attention recently. Low chlorinated PCBs and some hydroxylated metabolites display estrogenic properties (Arcaro *et al*. 1998; Arcaro *et al*. 1999; Bonefeld-Jorgensen *et al*. 2001; Connor *et al*. 1997; Fielden *et al*. 1997; Gierthy *et al*. 1997; Kramer *et al*. 1997; Krishnan & Safe 1993; Nesaretnam & Darbre 1997; Ramamoorthy *et al*. 1999; Ramamoorthy *et al*. 1997; Shekhar *et al*. 1997). Mono- and non-*ortho* substituted PCB congeners share some structure similarities with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and can bind to the aryl hydrocarbon (Ah) receptor. Dioxin-like compounds elicit a broad spectrum of antiestrogenic activities and may reduce breast cancer risk; the interaction between AhR/ARNT and the estrogen response element (ERE) has been proposed to be at least partially responsible for this antiestrogenic effect by AhR (Klinge *et al*. 1999). On the other hand, activation of the Ah receptor leads to induction of CYP1A1/2 and CYP1B1 expression, which in turn could increase the biotransformation of estradiol to catechol estrogens, the latter exhibiting genotoxic effects. Di-ortho-substituted congeners induce CYP2B1/2 and CYP3A4, similar to the activity of phenobarbital, and those enzymes are also involved in the metabolism of estradiol.

Most of epidemiological studies failed to show any association between exposure to total PCBs and breast cancer risk (Aronson *et al*. 2000; Davidson 1998; Dorgan *et al*. 1999; Helzlsouer *et al*. 1999; Holford *et al*. 2000; Hunter *et al*. 1997; Laden *et al*. 2001a; Laden *et al*. 2001b; Liljegren *et al*. 1998; Lucena *et al*. 2001; Moysich *et al*. 1998; Moysich *et al*. 1999; Safe 1997; Ward *et al*. 2000; Wolff *et al*. 1993; Wolff *et al*. 2000; Zheng *et al*. 2000a; Zheng *et al*. 2000b), but recent studies suggested that exposure to specific congeners may be related to breast cancer. A case-control study showed that exposure to dioxin-like PCBs (PCB 105, 118, and 156) increased breast cancer risk (Demers *et al*. 2002), but another study showed the opposite results, in which PCB-156 had a protective effect, while phenobarbital-like PCB-180 and –183 increased breast cancer risk (Holford *et al*. 2000).

PCBs and liver cancer

Although studies following the Yucheng population have shown an increase in mortality from chronic liver disease and cirrhosis (Hsieh *et al*. 1996; Yu *et al*. 1997), there is no direct evidence on PCBs-induced liver cancer from epidemiological studies. In the Yusho population, an increased incidence of hepatocellular carcinoma has been linked to the PCB exposure (Kikuchi 1984).

PCBs and pancreatic cancer

There has been no animal study examining the possible association between PCBs and pancreatic cancer. One study examining a cohort of workers once employed at a transformer manufacturing plant showed significantly increased mortality from pancreatic cancer, while the overall cancer death was not significantly increased (Yassi *et al*. 1994).

PCBs and skin cancer

An epidemiological study of cancer mortality among 138,905 electric utility workers showed increased mortality from malignant melanoma with PCB exposure (Loomis *et al*. 1997). Another epidemiological study showed a 4-fold increase in mortality from malignant melanoma in 3,588 electrical capacitor manufacturing workers with known PCB exposure (Sinks *et al*. 1992).

Experimental animals studies

PCBs have been suggested to have tumor promoting activity during carcinogenesis since the early rodent carcinogenicity studies in 1970's. Ito *et al.* found that coadministration of PCBs (Kanechlor 500) with benzene hexachloride (BHCs) in the diet produced more incidences of nodular hyperplasia and hepatocellular carcinoma than that produced by BHCs alone (Ito *et al*. 1973). Since that, the carcinogenicity of PCBs has been studied extensively in a variety of organs of rats and mice.

PCBs and breast cancer

Nesaretnam *et al*. have shown a promoting or co-carcinogenic effect of PCBs in rat mammary carcinogenesis (Nesaretnam *et al*. 1998). Rats were injected with 3,3'4,4'-

tetrachlorobiphenyl (PCB-77) following 7,12-dimethylbenz(a)anthracene (DMBA) initiation and then kept on diet containing PCB-77 for one week, rats were then kept on low- or high-fat diets with no PCB for 9 weeks. PCB-77 increased the number and incidence of mammary tumor, with the highest effect in animals fed a high fat diet.

PCBs and liver cancer

1. PCBs and liver tumor

The role of PCBs mixture or specific congeners in liver carcinogenesis has been studied extensively in rats and mice (Glauert 2001; Safe 1994; Silberhorn *et al*. 1990). The general view of PCB mixtures or congeners is that either of them can act as tumor promoters by increasing the number of tumor or by inducing clonal expansion of spontaneously or chemically initiated cells to form preneoplastic lesions.

Ito *et al*. demonstrated an enhanced tumor development in liver by PCBs when they were coadministrated with known hepatic carcinogen benzene hexachlorides (BHCs) (Ito *et al*. 1973). In the following studies, Nishizumi showed that administration of PCBs (Kanechlor-500) following treatment with hepatic carcinogen diethylnitrosamine (DEN) resulted in earlier appearance of tumors and a significant increase in the number of tumors (Nishizumi 1976). Study results did not show an acceleration of tumor growth.

Using a modified protocol including a partial hepatectomy (PH) three weeks after initiation by N-2-fluorenylacetamide (2-FAA), Tatematsu *et al*. showed an enhancing effect on induction of hyperplastic liver nodules by the co-administration of 2-FAA and PCBs (500 or 1000 ppm) in male F344 rats (Tatematsu *et al*. 1979). It was shown that 2-FAA or PH alone, or PCBs plus PH did not induce hyperplastic liver nodules, but 2-FAA plus PCBs significantly increased the number and total area of hyperplastic nodules. PH in the protocol after initiation by 2-FAA increased the tumorigenesis more than protocols without PH.

Using N-ethyl-N-hydroxyethylnitrosamine (EHEN) as initiator, Hirose *et al*. showed that administration of PCB (0.05% in diet) for 32 weeks following initiation by EHEN significantly increased the incidence of hepatocellular carcinomas in male F344 rats (Hirose *et al*. 1981). However, PCB failed to show any effect in renal carcinogenesis in the same study.

Preston *et al.* investigated the hepatic tumor promoting activity of Aroclor 1254, with DEN as the initiator (Preston *et al*. 1981). Administration of Aroclor 1254 (100 ppm in the diet) for 18 weeks following initiation significantly increased the incidence of hepatocellular carcinomas, while administration of Aroclor 1254 alone did not induce tumors. The removal of polychlorinated dibenzofuran (PCDF) impurity did not show any significant effect on the tumor promoting activity of Aroclor 1254.

The tumor promoting activity of PCB (Aroclor 1254) in suckling Swiss mice was investigated (Anderson *et al*. 1983). Aroclor 1254 was administrated i.p. to pregnant mice, the suckling offspring of these mice and controls were treated with DEN. The DEN treatment caused liver tumors, and exposure to PCB significantly increased the incidence of liver tumors at the age of 18 months.

Diwan *et al*. compared Aroclor 1254 to phenobarbital (PB) and dichlorodiphenyltrichloroethane (DDT) for their tumor promoting activities in D2B6F1 male mice initiated with N-nitrosodiethylamine (NDE) (Diwan *et al*. 1994). At the test doses, Aroclor 1254 (175 and 350 ppm in diet) was more efficacious then PB (500 ppm in diet) or DTT (300 ppm in diet) at increasing the multiplicity of hepatocellular carcinomas, but did not affect the incidence or multiplicity of hepatoblastomas.

2. Initiation/promotions studies using altered hepatic foci as marker

The ability of commercial PCB mixtures and individual congeners to initiate or promote the preneoplastic lesions has been studied using several different initiation-promotion hepatocarcinogenic protocols. Altered hepatic foci (AHF) are the most used endpoints to quantify preneoplastic lesions, since the development of these biochemical markers has been correlated with the later development of malignant neoplasms (Pitot & Dragan 1994). The most commonly used foci in PCB studies are those deficient in ATPase and those that contain increased placental glutathione S-transferase (PGST) or γ-glutamyl transpeptidase (GGT).

Mixtures of PCBs

An early study using AHF as markers to determine the tumor promoting activity of PCB was done by Deml and Oesterle (Deml & Oesterle 1982). The administration of Clophen A 50 (50 or 100 mg/kg body wt/week for 7 weeks) to Sprague-Dawley rats

following initiation by DEN significantly increased the number and volume of ATPase-deficient and GGT-positive foci, while Clophen alone only resulted in a small number of foci. The tumor promoting activity of PCBs was shown to be sex dependent, and PCBs showed more potent tumor promoting activity in female rats than in male rats. Another study done by Pereira *et al*. demonstrated that a single dose of Aroclor 1254 (500 mg/kg) promoted GGT-positive foci in rat liver after a 2/3 partial hepatectomy (PH) and initiation by DEN (Pereira *et al*. 1982).

Oesterle and Deml further investigated the tumor promoting activity of the PCB mixture Clophen 50 in weanling Sprague-Dawley rats using N-nitrosamine as initiator (Oesterle & Deml 1983). Administration of Clophen 50 (100 mg /kg of body weight, once a week for 1-7 weeks) following initiation caused an early appearance and increased number and total volume of ATPase-deficient and glycogen- and GGT-positive foci, while Clophen 50 alone caused few foci. The same group (Deml and Oesterle) observed a dose-dependent tumor promoting activity of Clophen A50 in weanling female Sprague-Dawley rats initiated with DEN (Deml & Oesterle 1987). Different levels of Clophen A50 (0.1, 0.5, 1.0, 5 and 10 mg/kg of body weight, three times per week for 11 weeks) were given to rats after a single dose of DEN. The lowest effective dose of Clophen A50 was shown to be 1 mg/kg body weight.

Denk *et al*. demonstrated the dose dependent effect of the initiating agent on PCB promotion (Denk *et al*. 1990). The male and female Sprague-Dawley rats were initiated by 2-nitropropan vapors (0, 25, 40, 50, 80, and 125 ppm, 6 h/day, 5 days/week for 3 weeks), and then were treated with Clophen A50 (10 mg/kg body weight, twice a week for 8 weeks). The number of ATPase-deficient foci was correlated with the dose of 2-nitropropan. Also, a sex-dependent effect was observed in this study, female rats exhibited an approximately four times higher incidence of foci than male rats.

Another initiating agent, aflatoxin B1 (AFB1), was used by Pelissier and coworkers to test the promoting activity of PCB mixture Phenoclor DP-6 (Pelissier *et al*. 1992). After initiation by AFB1, male Sprague-Dawley rats were fed a diet containing 50 ppm Phenoclor DP-6 for 11 days, and the number and volume of GGT-positive foci was increased by PCB administration.

Studies did not show any solid evidence that PCB mixtures could act as tumor initiator in liver. Pereira *et al.* showed that Aroclor alone did not initiate GGT-positive foci. Studies by Deml and Oesterle showed that PCB (Clophen) alone only resulted in a small number of foci, which could be the results of promotion of "spontaneous" foci or those initiated by "contaminants" in the PCB mixtures or diet.

Individual PCB congeners

A number of studies addressed the initiation/promotion of AHF by individual PCB congeners. Preston *et al*. have shown that both 2,2',5,5'-tetrachlorobiphenyl (PCB-52) and 2,2'4,4'-tetrachlorobiphenyl (PCB-47) (100 ppm in diet for 27 weeks) promoted GGT-positive foci after initiation by PH and DEN (Preston *et al*. 1985).

Kobusch *et al*. investigated the effects of 3,3'4,4'-tetrachlorobiphenyl (PCB-77) on the growth of glucose-6-phosphatase (G6Pase)-altered foci in B6C3F1 mice (Kobusch *et al*. 1989). Mice were initiated by N-nitrosomorpholine (NNM), and then received PCBs (five i.p. injections, 50 mg/kg body weight, every 3 days). The number of G6Pase-negative and positive foci was decreased by PCB, while the mean volume of foci was increased as the result of an increase in the percentage of foci of large size. The tumor promoting activity of PCB-77 was tested in other several studies. The initiators used included DEN (Berberian *et al*. 1995; Buchmann *et al*. 1986; Buchmann *et al*. 1991; Sargent *et al*. 1991; Tharappel *et al*. 2002), NNM (Wolfle *et al*. 1988), and PH plus DEN (Sargent *et al*. 1991); the markers used included ATPase-deficient foci (Berberian *et al*. 1995; Buchmann *et al*. 1986; Buchmann *et al*. 1991; Sargent *et al*. 1991; Wolfle *et al*. 1988), GGT-positive foci (Berberian *et al*. 1995; Buchmann *et al*. 1991; Sargent *et al*. 1991), and PGST-positive foci (Sargent *et al*. 1991; Tharappel *et al*. 2002). Data from all these studies demonstrated a promoting activity of PCB-77 on the growth of AHF.

A study of different PCB congeners, based on their ability to induce cytochrome P450, found that both a phenobarbital (PB)-type inducer (2,2'4,4',5,5'-hexachlorobiphenyl, PCB-153) and a 3-methylcholanthrene (3-MC)-type inducer (3,3'4,4'-tetrachlorobiphenyl, PCB-77) promoted ATPase-deficient focal lesion in female Wistar rats initiated with DEN (Buchmann *et al*. 1986). PCB-77 was more potent than PCB-153 in this study, although PCB-153 is poorly metabolized and thus has a long half-

life in the body, while PCB-77 is just the opposite. In this case, the persistence of a PCB did not determine its tumor promoting capacity.

Other studies on PCB-153 also show its promoting activity. Hemming *et al.* showed that PCB-153 (1000, 5000, or 20,000 μg/kg/week s.c. for 20 weeks) enhanced GGT-positive and PGST-positive focal lesions after initiation with PH and DEN (Hemming *et al*. 1993). PCB-153 has also been shown to promote PGST-positive focal lesion after initiation with a single dose of DEN (Berberian *et al*. 1995; Tharappel *et al*. 2002); the dose of PCB-153 used in both studies was 4 i.p. injections of 300 μmol/kg body weight every other week.

Other PCB congeners which have been studied include 4-chlorobiphenyl (Buchmann *et al*. 1991), 2,2'4,5'-tetrachlorobiphenyl (PCB-49) (Buchmann *et al*. 1991), 2,3,3'4,4'-pentachlorobiphenyl (PCB-105) (Haag-Gronlund *et al*. 1998; Hemming *et al*. 1993), 2,3,4,4',5- pentachlorobiphenyl (PCB-114) (Buchmann *et al*. 1991), 2,3',4,4',5-pentachlorobiphenyl (PCB-118) (Haag-Gronlund *et al*. 1997b; Haag-Gronlund M 2000), 3,3',4,4',5-pentachlorobiphenyl (PCB-126) (Dean *et al*. 2002; Haag-Gronlund *et al*. 1998; Haag-Gronlund *et al*. 1997b; Hemming *et al*. 1995; Hemming *et al*. 1993), and 2,3,3',4,4',5-hexachlorobiphenyl (PCB-156) (Haag-Gronlund *et al*. 1997a). All PCB congeners tested, except 4-chlorobiphenyl, have been shown to promote the growth of AHF.

The interactive effects between different PCBs have been investigated by co-administration of two PCBs together. Sargent *et al.* examined the separate and combined effect of non-coplanar 2,5,2',5'-tetrachlorobiphenyl (PCB-52) and coplanar 3,4,3',4'-tetrachlorobiphenyl (PCB-77) (Sargent *et al*. 1991). Rats were initiated with PH/DEN and then were fed the PCBs for one year. PCB-77 alone (0.1 ppm in diet) following initiation did not increase the number or volume of AHF, while PCB-52 (10 ppm in diet) increased the volume but not the number of AHF. The co-administration of the two PCBs, however, synergistically increased both the number and volume of PGST-positive and ATPase-negative focal lesions. Haag-Grolund and coworkers examined the interactive effects between a non-*ortho* substituted PCB (PCB-126), a mono-*ortho* substituted PCB (PCB-105), and a both-*ortho* substituted PCB (PCB-153) (Haag-Gronlund *et al*. 1998). In this study, rats were initiated with PH/DEN and then fed 15

systematically select dose combinations of PCBs for 20 weeks. No synergistic effects were observed in this study. An additive effect was observed between PCB-105 and PCB-153, while antagonistic effects were observed between PCB-126 and PCB-153, and between PCB-126 and PCB-105, respectively. Berberian *et al*. investigated the interactive effects between PCB-77 and PCB-153 (Berberian *et al*. 1995). In this study, each PCB was given to rats at a dose of 300 μmol/kg every two weeks for four injections, or 150 μmol/kg each for the combination of two PCBs, after a single dose of DEN. The co-administration of PCB-153 decreased the volume and number of foci induced by PCB-77. The same results were observed in a study using the similar protocol (Tharappel *et al*. 2002), in which the rats were initiated with a single dose of DEN and then received 4 i.p. injections of PCB-77 (100 or 300 μmol/kg) or PCB-153 (100 or 300 μmol/kg), or both PCBs (100 μmol/kg each) every two weeks. Both PCB-77 and PCB-153 increased the number and volume of PGST-positive foci, and antagonistic effects were observed between the two PCBs. van der Plas *et al*. compared the tumor promoting capacity of a mixture of polyhalogenated aromatic hydrocarbon (PAHs) with and without PCB-153. The PAH mixture contained 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 1,2,3,7,8-pentachlorodibenzo-p-dioxin (PeCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), PCB-118, PCB-156, and PCB-126. The rats were initiated with PH/DEN and then were promoted by s.c. injections of PAH mixtures with and without PCB-153 (once a week for 20 weeks). The addition of PCB-153 slightly but insignificantly increased the mean focal volume and the volume fraction of PGST-positive foci. A recent study demonstrated an antagonistic effect between PCB-126 and PCB-153 (Dean *et al*. 2002), in which the female F344 rats were treated with a single i.p. injection of DEN followed by PH and by oral administrations of PCB126 (0.1, 1.0 and 10 μg/kg body weight) or PCB-153 (10, 100, 1000, 5000, and 10000 μg/kg body weight), or the combination of two PCBs (0.1 + 10, 1 + 100, 10 + 1000, 10 + 5000, and 10 + 10000 μg/kg body weight) 3 times weekly for 6 weeks. Each PCB alone caused a dose dependent increase in the number and volume of PGST-positive foci, while treatment with the mixture of two PCBs resulted in an antagonistic PGST-positive focal formation at all dose combinations.

Based on all studies, we can draw a few conclusions on the roles of PCBs in liver carcinogenesis. Both PCB mixtures and individual congeners have tumor promoting

activity; the PCB congeners include coplanar PCBs (PCB-77 and PCB-126), di-*ortho* substituted PCBs (PCB-47, PCB-49 and PCB-153), and mono-*ortho* substituted PCBs (PCB-105, PCB-114, PCB-118, and PCB-156), but a lower chlorinated PCB (4-chlorobiphenyl) is a poor promoter. DEN is the most common used initiator in the promotion study of PCB in rodent livers, but other initiating agents used include NNM and aflatoxin B1. Generally, there is no synergistic effect between PCB congeners.

PCBs and lung cancer

PCBs mixtures and specific congeners have been shown to promote lung tumor formation in rodents. The first evidence of PCBs as a lung tumor promoter was shown by Anderson *et al*., in which a single dose of Aroclor 1254 significantly increased the incidence of lung tumors (alveologenic adenomas) in N-nitrosodimethylamine (NDMA)-initiated infant mice, while Aroclor alone had no effect (Anderson *et al*. 1986). In the following study by the same group, 2,2',3,4,4',5'-hexachlorobiphenyl , an Ah-receptor agonist, promoted lung tumors in NDMA-initiated infant mice, while 2,2',4,4',5,5'-hexachlorobiphenyl, did not show promoting activity (Anderson *et al*. 1991). Aroclor 1254 showed a promoting activity on lung tumor formation in NDMA-initiated mice (Anderson *et al*. 1994).

The specific sex and initiator-related differences in PCBs-induced lung tumorigenesis have been investigated. Aroclor 1254 increased the incidence of lung tumors initiated transplacentally by NDMA or 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in male mice (Beebe *et al*. 1993), but not in female mice. In mice initiated neonatally, Aroclor 1254 promoted NNK-initiated lung cancer in females, and NDMA-initiated lung cancer in males. Kanechlor-400 has been shown to promote 1-nitropyrene-initiated lung tumorigenesis in mice (Nakanishi *et al*. 2001).

One study showed the activation of *ras* oncogene by PCBs (Ramakrishna & Anderson 1998). In this study Aroclor 1254 affected the level and membrane location of the K-*ras* p21 protein.

One early study showed the protective effect of PCBs mixture Aroclor 1254 in lung cancer prior to NDMA treatment in mice (Anderson *et al*. 1983), but in the same study, Aroclor increased the NDMA-initiated liver cancer.

PCBs and skin cancer

Animal studies using 2,2',5,5'-tetrachlorobiphenyl or 2,2',5,5'-tetrachlorobiphenyl 3,4-oxide failed to show promoting activity for these agents in mouse skin carcinogenesis (Preston *et al*. 1985), although they did show promoting activity for hepatic GGT-positive foci initiated with diethylnitrosamine.

In summary, both PCB mixtures and specific congeners have been demonstrated to have tumor promoting activities in liver and lung. There is very limited data on the relationship between PCB exposures and cancer in other organs.

B. Mechanisms of PCBs as tumor promoter

Induction of cytochrome P-450 by PCBs

PCBs as inducers of cytochrome P-450 mono-oxygenases have been studied extensively. PCB congeners can be divided into three categories based on their structure-activity relationships.

1. Co-planar PCB congeners

Coplanar PCBs are PCB congeners that are substituted in both *para* and at least two *meta* positions but not in any of the *ortho* positions, they may form a coplanar configuration similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and bind strongly to Ah receptor. The toxic effects of coplanar PCBs include immunological and reproductive effects, the induction of a wasting syndrome, hepatic toxicities such as hepatimegaly, the induction of cytochrome P4501A1 and 1A2 (CYP1A1, CYP1A2) and carcinogenesis.

2. Non-coplanar PCB congeners

PCB congeners with chlorine substitutes in two *ortho*-positions are non-coplanar, have low affinity to Ah receptor; they induce the cytochrome P450 2B1 and 2B2 (CYP2B1, CYP2B2), in a pattern similar to phenobarbital (PB), and some of them are neurotoxic (Fischer *et al*. 1998). The induction of CYP2B1 by the non-coplanar PCBs is mediated by the constitutive androstane nuclear receptor (CAR) (Muangmoonchai *et al*. 2001). Like PB, these PCBs can have hepatic tumor promoting activity.

3. Mixed-type PCB congeners

Mixed-type PCB congeners are those which have the features of both coplanar and non-coplanar PCB congeners. Usually they contain only one *ortho*-substituted chlorine and they bind to the AhR at a lower affinity than do the coplanar PCB congeners.

Liver carcinogenesis and tumor promoter

Liver carcinogenesis can be divided into at least three consecutive phases: initiation, promotion, and progression (Dragan *et al*. 1993). The first step, initiation, results from a genetic change, which could be due to a chemical, physical, or microbial agent. The genetic alteration is irreversible; however, the initiated phenotype is not fully expressed unless the cell is promoted, which is the second step of carcinogenesis. The promotion stage involves a reversible clonal expansion of single initiated cells. A fraction of hepatic focal lesions represent the precursor to hepatic cancer in the evolution of hepatocellular carcinoma. Progression is the final stage of carcinogenesis and occurs when a neoplasm develops into a benign or a malignant neoplasm. Tumor promoters are a group of compounds that can enhance the growth of hepatic focal lesions by changing cell proliferation and cell death. Tumor promoters, such as phenobarbital and peroxisome proliferators, do not interact directly with genomic DNA, but chronic administration of these compounds cause the increased incidence of hepatic tumors (Bayly *et al*. 1994; Cunningham 1996; Gill *et al*. 1998; Roberts *et al*. 1995; Shane *et al*. 2000). The exact mechanisms by which tumor promoters exert their promoting activities are still unknown. Mechanisms including an inhibition of apoptosis and induction of cell proliferation have been proposed for the change in growth of hepatic focal lesion caused by tumor promoters. The possible intermediate steps on the pathways for PCBs to hepatotumorigenicity were suggested to include PCBs accumulation in the liver, development of oxidative stress, and promotion of hepatic foci lesion growth.

Reactive oxygen species and cancer

Reactive oxygen species (ROS) include superoxide anion, hydrogen peroxide, and the hydroxyl radical. ROS are generated in cells because of incomplete reduction of molecular oxygen to water during aerobic metabolism. Usually, cellular antioxidants and

the endogenous antioxidant enzymes inactivate ROS to maintain an intracellular reduction and oxidation (redox) balance. Inflammation, hyperoxia, ultraviolet (UV) and ionizing irradiation, and certain oxidant chemicals induce over-production of ROS. The generation of excess ROS has been proposed as a mechanism by which some xenobiotics produce cancer (Cerutti & Trump 1991; Huber *et al*. 1991; Hunt *et al*. 1998). Compounds can produce ROS through biotransformation into reactive intermediates and lead to genotoxic events such as DNA strand breaks or oxidative DNA damage (e.g. 8-hydroxyguanosine). In addition to reacting with important macromolecules (lipids, proteins, DNA), ROS can stimulate changes in gene expression, such as the overexpression of c-*fos*, c-*jun*, and c-*myc* (Crawford 1990). The transcription factors activator protein 1 (AP-1) and nuclear factor-κB (NF-κB) are stimulated by ROS (Abate *et al*. 1990; Li & Karin 1999), which can lead to enhanced cell proliferation. Protein kinase C (PKC), another regulator of hepatocyte proliferation, also is stimulated by ROS (Das & White 1997). In addition, oxidative stress blocks gap junction intercellular communication, an important mechanism of tumor promotion and cell proliferation (Hotz-Wagenblatt & Shalloway 1993).

Oxidative stress by PCBs

Increased oxidative stress is one of mechanisms by which PCBs may exert their tumor promoting activity. PCBs cause increases in oxidative stress through a number of mechanisms. PCBs, especially the higher chlorinated congeners, selectively induce cytochrome P-450s (Parkinson *et al*. 1983) that may catalyze the oxidation of a broad range of endogenous and exogenous chemicals, including PCBs themselves. This induction of specific P-450 isozymes is through ligand-receptor interaction, including Ah receptor and CAR, as discussed before. In addition, non-coplanar PCBs also induce cytochrome P-450 3A genes through the pregnane X receptor (PXR) (Schuetz *et al*. 1998). Reactive oxygen species (ROS) in the form of superoxide or hydrogen peroxide can "leak" out of the cytochrome P-450 system (Goeptar *et al*. 1995), which then increases the level of oxidative stress. For low chlorinated PCBs, their metabolism is mediated primarily through cytochrome P-450s, which catalyze the oxidation of chlorinated biphenyls to mono- and di-hydroxy metabolites. The di-hydroxy metabolites

can autoxidize or can be enzymatically oxidized into semiquinones and/or quinones (McLean *et al*. 1996); these quinone metabolites participate in redox cycling, with the formation of ROS, thus being a source of oxidative stress (McLean *et al*. 2000). Production of ROS by PCB dihydroxyl metabolites has been shown to increase the levels of DNA strand breaks (Srinivasan *et al*. 2001). The formation of ROS following PCBs exposure has been detected in cell-free systems (McLean *et al*. 1996; Oakley *et al*. 1996), in cell cultures (Slim *et al*. 2000), and in experimental animals (Dogra *et al*. 1988; Kamohara *et al*. 1984; Pelissier *et al*. 1990; Pereg *et al*. 2002; Saito 1990; Twaroski *et al*. 2001a; Twaroski *et al*. 2001b). Biomarkers of PCBs-induced oxidative stress include lipid peroxidation (Kamohara *et al*. 1984; Pelissier *et al*. 1990; Peltola *et al*. 1994; Saito 1990), DNA-single strand breaks (Srinivasan *et al*. 2001), a decrease in antioxidant defense (Schramm *et al*. 1985; Srinivasan *et al*. 2001; Twaroski *et al*. 2001b), and activation of the oxidative stress-sensitive transcription factors NF-κB and AP-1 (Tharappel *et al*. 2002; Twaroski *et al*. 2001a).

Several studies have shown an increase in hepatic lipid peroxidation after PCBs exposure. The PCB mixture Kanechlor 500 (500 mg/kg diet for 5 months) caused an increase in hepatic thiobarbituric acid reactive substances (TBARS) (Kamohara *et al*. 1984). Aroclor 1248 administration (100 mg/kg diet for 240 days) caused an increase in hepatic TBARS, and this increase was prevented by higher level of dietary vitamin E. Saito showed that the administration of PCB (0.05% in the diet for 10 days) caused an increase in the TBARS in liver homogenates as well as in the cytosolic, mitochondrial, and microsomal fractions, but not the nuclear fraction (Saito 1990). Yamamoto *et al.* also reported the increased TBARS in hepatic microsomal and mitochondrial fractions after PCB exposure (0.02% in the diet for 10 days), and this increase was prevented by dietary vitamin E (0.1 %) or probucol (Yamamoto *et al*. 1994). Dogra *et al*. examined the production of ethane and the concentration of hepatic malondialdehyde (MDA) in male Sprague Dawley rats after a single dose of Aroclor 1254 (500 mg/kg) or one of the three PCB congeners (300 μmol/kg): PCB-77, PCB-114, or PCB-153 (Dogra *et al*. 1988). Total ethane exhalation was increased during the 30-day period after Aroclor 1254 administration, but this increase was due to an inhibition of ethane metabolism during the first two weeks, while it was due to increases in ethane production during the third and

fourth weeks. Hepatic MDA concentration was increased by Aroclor 1254 after 30 days, but not after 1, 3, 5, 7, or 14 days. Also, increases in hepatic MDA and ethane production rates were found 30 days after administration of single dose of one of three congeners.

Studies also showed that specific PCB congeners could cause increases in lipid peroxidation. Our lab recently found that hepatic TBARS concentration was increased 2, 6, or 10 days after a single dose of either PCB-77 or PCB-153 (150 μmol/kg) in male Sprague Dawley rats (Fadhel *et al*. 2002). Recently, Hassoun *et al.* have shown that 13 weeks exposure of PCB-126 produced dose-dependent increases in the production of superoxide anion, TBARS, and DNA single-strand break in rat hepatic and brain tissues (Hassoun *et al*. 2001). The same group reported the same increases in these biomarkers after 30 weeks of PCB-126 (Hassoun *et al*. 2002).

Lipid peroxidation may affect carcinogenesis through a number of mechanisms. The lipid peroxidation product 4-hydroxy-2-nonenal (HNE) can induce cell proliferation (Watanabe *et al*. 2001). Some of lipid peroxidation products, such as MDA, can react with DNA and thus are genotoxic (Basu & Marnett 1984; Benamira *et al*. 1995), and are carcinogenic in rats (Dedon *et al*. 1998). Also, cell proliferation and/or apoptosis could be affected by the oxidation products of polyunsaturated fatty acid could by acting on signal transduction pathways (Colquhoun & Schumacher 2001; Welsch 1995).

Another mechanism by which oxidative stress from PCB exposure could affect tumor promotion is through alterations in gene expression. The Ah receptor, CAR and PXR-dependent induction of cytochrome P-450 have already been discussed. In addition, the oxidative stress-sensitive transcription factor NF-κB may be involved in this pathway.

NF-κB in liver carcinogenesis

Recently, hepatic tumor promoters such as peroxisome proliferators, phenobarbital, and specific PCB congeners (PCB-77 and PCB-153) have been found to activate transcription factors (e.g. NF-κB) that regulate the expression of cell proliferation- and apoptosis-related genes (Li *et al*. 2000; Li *et al*. 1996a; Li *et al*. 1996b; Nilakantan *et al*. 1998; Tharappel *et al*. 2002). The activation of NF-κB has been found to be mediated by active oxygen (Abate *et al*. 1990; Amstad *et al*. 1992; Schreck *et al*. 1992), and NF-κB has been considered as a sensitive biomarker of oxidative stress (Li &

Karin 1999). Constitutive activation of NF-κB has been discovered in certain types of carcinoma (Dejardin *et al*. 1995; Sovak 1997; Tai *et al*. 2000).

NF-κB is present in cytoplasm as an inducible heterodimer and usually consists of two subunits, a 50-kDa (p50) and a 65-kDa (p65) polypeptide that are complexed to an inhibitory subunit (IκB). Upon stimulation, IκB is phosphorylated, ubiquinated and degraded; NF-κB then can translocate into the nucleus, bind to the κB-binding site in promoter region of certain genes and alter gene expression. The role of NF-κB in cell growth and tumorigenesis has been studied extensively because of the ability of NF-κB to modulate transcription of genes whose products are involved in cell proliferation and suppression of apoptotic cascade. Among the NF-κB-regulated genes, cyclin D1 controls the cell cycle checkpoint at G1/S phase (Guttridge *et al*. 1999; Hinz *et al*. 1999). NF-κB is required for the initiation of cyclin D1 transcription and hyperphosphorylation of pRB, which lead cells to progress through G1 and entry into S phase (Biswas *et al*. 2000; Henry *et al*. 2000; Joyce *et al*. 1999; Kaltschmidt *et al*. 1999). The overexpression of cyclin D1 has been observed in hepatocellular carcinoma (HCC) (Joo *et al*. 2001); studies with transgenic mice model show that targeted overexpression of cyclin D1 leads to the development of mammary carcinomas (Wang *et al*. 1994) and hepatocellular carcinoma (Deane *et al*. 2001). Hepatocyte cyclin D1 expression can be increased by the hepatic tumor promoters nafenopin (Chevalier & Roberts 1999) and phenobarbital (Kinoshita *et al*. 2002), which suggests that changes in the cyclins/CDK complexes and their inhibitors could result in the loss of regulation of hepatocyte proliferation.

Alteration of cell apoptosis is another mechanism by which non-genotoxic carcinogens may induce cancer. Dysregulation of apoptosis may result in the decreased ability of cell to undergo apoptosis and provide a selective survival advantage for those cells, thus leading to altered growth, cellular transformation and tumor progression. The balance between cell proliferation and death determines the fate of cells in hepatocarcinogenesis, such as cell growth and clonal expansion (Kolaja *et al*. 1996). Tumor promoters such as phenobarbital and WY-14,643 have been shown to reduce relative rates of cell death in both normal liver and hepatic neoplasia (Kolaja *et al*. 1996). Withdrawal of these tumor promoters resulted in regression of hyperplastic liver, preneoplastic foci, and adenomas with an increase in apoptosis. Besides, phenobarbital

has been shown to inhibit the spontaneous or transforming growth factor β1 (TGF-β1)-induced apoptosis in primary hepatocyte culture and mouse liver (Sanders & Thorgeirsson 1999; Sanders & Thorgeirsson 2000). These studies indicate that apoptosis plays an important role in rodent hepatocarcinogenesis.

The molecular targets involved in the regulation of apoptosis have been studied to elucidate the alteration of apoptosis during hepatocarcinogenesis. NF-κB regulates expression of certain anti-apoptotic genes, such as cIAPs (Wang *et al*. 1998) and Bcl-2/Bcl-x_L (Lee *et al*. 1999; Tamatani *et al*. 1999; Wang *et al*. 1999). Knockout studies revealed that the p65 subunit of NF-κB is essential for cell to survive the TNF-α induced apoptosis, because deficiency in p65 is embryonic lethal (Beg *et al*. 1995). Human and murine Bcl-x_L promoters contain several κB-binding sites and the binding of NF-κB to the κB sites regulates the expression of Bcl-x_L and the cell resistant to apoptosis (Khoshnan *et al*. 2000; Lee *et al*. 1999). Bcl-2/Bcl-x_L locates in the outer membrane of mitochondrial and inhibits the release of cytochrome c; other members include apoptosis-inducing protein (e.g. Bax and Bak) and inhibiting proteins (e.g. BAG-1). Bcl-2 family members have been found to be involved in the inhibition of apoptosis by tumor promoters, such as phenobarbital and WY-14,643, in primary hepatocyte and in mouse liver (Christensen *et al*. 1998; Christensen *et al*. 1999; Sanders & Thorgeirsson 2000).

All of these studies suggest that NF-κB could potentially contribute to the nongenotoxic carcinogen-induced carcinogenesis through increasing cell proliferation and inhibition of apoptotic cell death.

Mouse deficient in the p50 subunit of NF-κB: a model to study the role of NF-κB in PCBs-induced carcinogenic process

Although p65 knockout mice die during embryogenesis (Beg *et al*. 1995), p50 -/- knockout mice are viable (Sha *et al*. 1995). Based on the study by DeAngelis et al., there is no noticeable difference in liver regeneration after a PH, or in liver repairing functions (hepatocyte DNA synthesis, mitosis and serum enzyme levels) after treatment with CCl_4 between the p50-/- mice and wild type mice (DeAngelis *et al*. 2001). In this study, a decrease in the protein and mRNA levels of IκBα and an increase in the protein level of p65 subunit were observed in the p50-/- livers, which could provide a compensation for

the loss of p50. The known differences between livers of p50-/- mice and wild type mice are: the p50-/- livers showed more apoptosis than livers of wild type mice after treatment with Fas antibody, and the levels of interleukin-6 (IL-6) were reduced in p50-/- livers but still adequate to support liver regeneration. Studies in our laboratory have shown that peroxisome proliferator ciprofibrate increased hepatocyte proliferation in both wild type mice and p50-/- mice, but the increase in p50-/- mice was less than that in the wild type mice (Tharappel 2001). In addition, p50-/- livers showed more apoptosis than the livers of wild type mice, apoptosis was reduced in p50-/- mice by treatment with ciprofibrate but was still higher than that in wild type mice. Thus, the p50-/- mice provide us a useful model to study the role of p65/p50 dimer (NF-κB) in the changes of cell proliferation and cell apoptotic death in response to treatment with PCBs.

3. Rationale, Hypothesis and Specific Aims:

A. Study Rationale and Hypothesis:

In light of the fact that PCBs have been shown to cause oxidative stress, and the fact that other tumor promoters such as phenobarbital and peroxisome proliferators induce activation of NF-κB, the major focus of the present study is to test the hypothesis that specific PCB congeners can activate NF-κB, which, in turn, can cause an increase in cell proliferation and/or inhibition of cellular apoptotic cascade. Also, the levels of dietary antioxidants will affect PCBs-induced NF-κB activation, which will cause an effect on the tumor promoting activities of specific PCB congeners.

B. Specific Aims:

The generation of reactive oxygen species has been a proposed mechanism by which some xenobiotics produce cancer. Studies have indicated that hepatic tumor promotion is an imbalance between cell proliferation and apoptosis. The transcription factor NF-κB is stimulated by oxidative stress and can lead to cell proliferation. We hypothesize that oxidative stress plays an important role in PCBs-induced tumor promotion, through the activation of NF-κB. To test this hypothesis, we proposed the following specific aims:

Specific Aim 1:

Determine the effects of selected PCB congeners on the hepatic NF-κB activation and cell proliferation.

The data in chapter 2 shows that a single dose of non-coplanar PCB congener PCB-153 caused hepatic NF-κB activation and cell proliferation in rat liver, while PCB-77, a coplanar PCB congener, showed no effects on NF-κB activation or cell proliferation.

Specific Aim 2:

Determine the roles of NF-κB in PCBs-altered hepatocyte proliferation and apoptosis. A p50 knockout mouse model was used in chapter 3 to examine effect of the absence of p50 subunit on the cell proliferation and apoptosis in the liver. The data in chapter 3 show an important role of NF-κB in the regulation of cell proliferation and apoptosis. A single dose of PCB-153 caused NF-κB activation and an increase in cell proliferation in wild type liver, but not in p50-/- livers. Cell proliferation was increased in p50-/- livers by multiple doses of PCB-153 after a longer exposure (21 days), but it was still lower than that in the wild type livers. Comparison of the mRNA levels of cyclin A2, B1, B2, C, D1 and D2 failed to show any difference between p50-/- and wild type livers, but I observed a decrease in the protein level of cyclin D1 in the p50-/- livers, as compared to the wild type livers. In addition, p50-/- livers showed a higher incidence of spontaneous apoptosis than wild type livers, and PCB-153 inhibited apoptosis in the p50-/- livers.

Specific Aim 3:

To determine the effect of dietary vitamin E (α-tocopherol acetate) on PCBs-induced hepatic focal lesion growth and cell proliferation during the promotion stage, and the effect of dietary vitamin E on PCBs-induced hepatic NF-κB activation. Data from chapter 4 show that both PCB-77 and PCB-153 induced the number and volume of placental glutathione S-transferase (PGST)-positive foci after diethylnitrosamine (DEN) initiation. Both vitamin E deficiency (10 ppm) and supplementation (250 ppm) caused an insignificant increase in the number and volume of PGST-positive foci compared to rats

received sufficient vitamin E (50 ppm). For hepatocyte proliferation, PCB-77 significantly increased cell proliferation in PGST-positive and –negative hepatocytes in low (10 ppm) and medium (50 ppm) vitamin E groups, the high level of dietary vitamin E (250 ppm) inhibited cell proliferation in both normal and PGST-positive hepatocytes, with more pronounced effect on normal hepatocytes. It was also observed that PCB-77 caused hepatic NF-κB activation, and the high level of vitamin E showed an insignificant inhibitory effect on NF-κB activation by PCB-77.

Chapter 2. Increased hepatic NF-κB DNA binding activity and cell proliferation by a single dose of polychlorinated biphenyls (PCBs) in rats

Introduction

PCBs cause several of toxic effects in liver, including liver hypertrophy, neoplastic nodules, and hepatocellular carcinoma (Safe 1994). PCBs have been shown to enhance cancer formation in animals after initiated with a genotoxic reagent. Thus, PCBs and related compounds induce cancer as tumor promoters (Glauert 2001; Safe 1994; Silberhorn *et al*. 1990). The mechanisms of the promoting activity of PCBs, however, have not been determined. Other hepatic tumor promoters, such as phenobarbital and peroxisome proliferators, act by suppressing apoptosis and/or increasing cell proliferation (Bayly *et al*. 1994; Cunningham 1996; Gill *et al*. 1998; Roberts *et al*. 1995; Shane *et al*. 2000). These changes are brought about by changes in the expression of genes related to cell proliferation or apoptosis. Another theory of tumor promotion is by oxidative stress, reactive oxygen species (ROS) in the form of superoxide or hydrogen peroxide can be released as a by-product from cytochrome P-450 and could contribute to lipid peroxidation (Goeptar *et al*. 1995). Other sources of oxidative stress may include other induced enzymes, the repression of antioxidants or antioxidant enzyme. Formations of ROS during PCBs exposure and metabolism have been detected in cell-free systems (McLean *et al*. 1996; Oakley *et al*. 1996), in cell cultures (Slim *et al*. 2000), and in laboratory animals (Dogra *et al*. 1988; Kamohara *et al*. 1984; Pelissier *et al*. 1990; Pereg *et al*. 2002; Saito 1990; Twaroski *et al*. 2001a; Twaroski *et al*. 2001b). Biomarkers of PCBs-induced oxidative stress include lipid peroxidation (Kamohara *et al*. 1984; Pelissier *et al*. 1990; Peltola *et al*. 1994; Saito 1990), DNA-single strand break (Srinivasan *et al*. 2001), and decrease in antioxidant defense (Peltola *et al*. 1994; Twaroski *et al*. 2001b).

Recently, hepatic tumor promoters such as peroxisome proliferators and phenobarbital have been found to activate transcription factors (e.g. NF-κB) that regulate the expression of cell proliferation- and apoptosis-related genes (Li *et al*. 2000; Li *et al*. 1996a; Li *et al*. 1996b; Nilakantan *et al*. 1998). The activation of NF-κB and AP-1 has

been found to be mediated by active oxygen (Abate *et al*. 1990; Amstad *et al*. 1992; Schreck *et al*. 1992). NF-κB has been considered as a sensitive biomarker of oxidative stress (Li & Karin 1999).

NF-κB is present in cytoplasm as an inducible heterodimer and usually consists of two subunits, a 50-kDa (p50) and a 65-kDa (p65) polypeptide that are complexed to an inhibitory subunit (IκB). Upon stimulation, IκB is phosphorylated, ubiquinated and degraded; NF-κB then can translocate into the nucleus, bind to the κB-binding site in promoter region of target genes and alter their expression. These target genes include those that are involved in cell proliferation, including cyclin D1, and others that are anti-apoptotic, such as cIAPs and Bcl-2/Bcl-xL (Guttridge *et al*. 1999; Hinz *et al*. 1999) (Lee *et al*. 1999; Tamatani *et al*. 1999; Wang *et al*. 1999; Wang *et al*. 1998). Knockout studies revealed that the p65 subunit of NF-κB is essential for cell to survive the TNF-α induced apoptosis, because deficiency in p65 is lethal (Beg *et al*. 1995).

The main goal in this study was to explore mechanisms by which PCBs may exert their tumor-promoting activities. We therefore examined the effect of PCBs on the induction of oxidative stress in the form of activation of the transcription factors NF-κB and AP-1, and the effect of PCBs on cell proliferation. The PCBs used in this study were 3,3',4,4'–tetrachlorobiphenyl (PCB-77) and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB-153); these two congeners were used because of their different chemical structures and abilities to induce different cytochrome P-450s (Safe 1994) (Tharappel *et al*. 2002).

Materials and Methods

Materials: 3,3',4,4'–tetrachlorobiphenyl (PCB-77) and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB-153) were synthesized and characterized as described previously (Schramm *et al*. 1985). Vitamin E stripped corn oil was obtained from Acros Organics (Morris Plains, NJ). Alzet osmotic pumps (model 2ML1) were obtained from Alza Scientific Products, Palo Alto, CA. The anti 5-bromo-2'-deoxyuridine (BrdU) antibody was purchased from Becton-Dickinson (San Jose, CA). The Antigen Retrieval Citra solution was purchased from BioGenex (San Ramon, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Experimental design and animal treatment: Male Sprague-Dawley rats weighing 200 grams were obtained from Harlan Sprague Dawley (Indianapolis, IN) and housed three rats per cage in a temperature and light-controlled room. The rats were allowed to acclimatize for one week before starting the experiment. In the first study, the rats were injected intraperitoneally (i.p.) with corn oil (10 ml/kg), PCB-153 (30 or 150 μmol/kg), PCB-77 (30 or 150 μmol/kg), or both PCBs (30 μmol/kg each) dissolved in corn oil. Rats (3 per group) were killed 2, 6, or 24 hours, or 2, 6 or 10 days after the PCB injection. For all but the 10-day time point, rats were injected subcutaneously (s.c.) with 5-bromodeoxyuridine (BrdU, 100 mg/kg) two hours before euthanasia. For the 10-day time point, rats were implanted with Alzet osmotic pumps containing BrdU (20 mg/ml, 10 μl/hr) 48 hours before euthanasia. In the second study, rats were injected i.p. with corn oil, PCB-77 (150 or 300 μmol/kg), PCB-153 (150 or 300 μmol/kg), or both PCBs (150 μmol/kg each). The rats (5 per group) were euthanized 2 or 6 days after the PCB injection; two hours before euthanasia all rats were injected s.c. with 5-bromodeoxyuridine (BrdU, 100 mg/kg). In both studies, liver pieces were removed and fixed in 10% formalin; paraffin sections were then stained with hematoxylin and eosin for histopathological analysis or immunohistochemically stained for BrdU for the analysis of DNA synthesis. The remainder of the liver was frozen in liquid nitrogen.

BrdU immunohistochemical staining:

Principle: BrdU is a synthetic analog of thymidine and can be incorporated into the DNA during replication (S phase). Immnuohistochemical techniques using a monoclonal antibody specific against BrdU allow for easy detection of BrdU incorporated nuclei, which indicates the relative rate of cell proliferation.

Reagents:

1. Hemo-De (Scientific Safety Solvent 15-182-507A)
2. PBS (phosphate buffered saline), pH 7.4

   For 4 liters:

   1.05 g sodium phosphate (monobasic)

   4.51 g sodium phosphate (dibasic)

   32 g sodium chloride (Sigma Chemical Co. S3014)

3. 95% Ethanol
4. H_2O_2 solution (make fresh)

   180 ml methanol + 20 ml 30% H_2O_2 (Sigma Chemical Co. H0904)
5. 1 x Antigen Retrieval Citra

   10 x Antigen Retrieval Citra (BioGenex HK086-5K) diluted 10 time with distilled water before use.
6. 10 x Automation Buffer (Biomedia Corp. M30)
7. Diluted normal horse serum

   3 drops of normal horse serum (Vector Laboratories Peroxidase Kit PK-6102)

   10 ml 1 x automation buffer
8. Blocking Kit (Vector Laboratories SP-2001)

   Contains Avidin solution and Biotin solution
9. Diluted primary antibody (anti-BrdU)

   25 μl of antibody (Becton Dickinson 347580)

   975 μl 1 x automation buffer
10. Diluted biotinylated anti-mouse IgG

    1 drop of biotinylated anti-mouse IgG (Vector Laboratories Peroxidase Kit PK-6102)

    3 drops of diluted normal horse serum

    10 ml 1 x automation buffer
11. ABC solution

    3 drops of A (Vector Laboratories Peroxidase Kit PK-6102)

    3 drops of B (Vector Laboratories Peroxidase Kit PK-6102)

    10 ml 1 x automation buffer

    allow solution to stand at room temperature for 20-30 minutes before use
12. DAB staining solution (make fresh)

    1 drop of buffer (Vector Laboratories Peroxidase Substrate Kit SK-4100)

    2 drops of DAB

    1 drop of H_2O_2 solution

    2.5 ml distilled water
13. 1% acid alcohol

    140 ml absolute ethanol

60 ml distilled water

2 ml HCl (Sigma Chemical Co. H7020)

14. Ammonium hydroxide solution

    200 ml distilled water

    10-15 drops of ammonium hydroxide (J. T. Baker Chemical Co. 9721)

15. Harris hematoxylin solution (Sigma Chemical Co. HHS-32)

Procedures:

1. The slides were dried in 60 °C oven for 30 minutes.
2. The slides were de-paraffined in Hemo De for a total of three changes (15 minutes 1st and 5 minutes for 2nd and 3rd change).
3. The slides were placed in 100% ethanol for 2 changes (5 minutes each change).
4. The slides were dipped in 95% ethanol for 5 minutes.
5. The slides received 3 rinses with tap water.
6. The slides were placed in the methanol:hydrogen peroxide solution (made fresh) for 10 minutes.
7. The slides received 3 rinses with tap water.
8. While being submerged in 1x antigen retrieval citra, the slides were heated in a microwave for 15 minutes in 5-minute intervals. Caution was taken in making sure the sections were always covered with solution.
9. The slides were cooled in the citra for 20 minutes.
10. The slides were rinsed with tap water 3 times.
11. Diluted Normal Horse Serum was added to the slides for 10 minutes at room temperature (3 drops/slide).
12. The horse serum was drained and the Avidin Block (undiluted) was added to the slides for 15 minutes (2 drops/slide).
13. The slides were dipped briefly in PBS and Biotin Block was added to the slides for 15 minutes at room temperature (2 drops/slide).
14. The slides were drained (not rinsed) and the diluted anti-BrdU primary antibody was added to the slides for 30 minutes at 37 °C (100 µl/slide using a 1:40 dilution of BrdU).
15. The slides were rinsed 2 times with PBS (5 minutes each).

16. Two drops of diluted Biotinylated Anti-mouse IgG were added to each slide for 15 minutes at 37 °C.
17. The slides were rinsed twice with PBS (5 minutes each).
18. Two drops of the ABC solution was applied to each slide for 15 minutes at 37 °C (3 drops A, 3 drops B, 10 ml 1x Automation Buffer – let stand 20-30 minutes before use).
19. The slides were rinsed with PBS twice (5 minutes each).
20. The DAB staining solution was added to each slide for 2-10 minutes at room temperature (enough to completely cover the sectioned tissue). This solution was made fresh, and each slide was monitored using a microscope to determine when staining was complete.
21. The slides were gently rinsed in tap water.
22. The slides were dipped in Hematoxylin for 1 minute. This solution was filtered daily with No. 1 Whatman Filter Paper.
23. The slides were dipped in tap water.
24. The slides were quickly dipped in 1% acid alcohol solution.
25. The slides were dipped several times in tap water.
26. Three dips were made in the ammonium hydroxide solution.
27. The slides were gently rinsed in running tap water for 5 minutes.
28. In order to dehydrate the slides, they were dipped 10 times in 95% ethanol, 100% Ethanol (3 jars) and Hemo-De (3 jars) for a total of 70 dips.
29. The slides were mounted with Permount medium (Fisher Scientific Co. SP15-500) and covered with cover slips (Clay Adams 3322). The slides were allowed to air dry before counting nuclei.

Counting:

1. Cells that had incorporated BrdU were easily identified with brown nuclei. At least 3000 hepatocellular nuclei were counted randomly per slide.
2. Labeling index (%) = $\left(\frac{\text{number of labeled nuclei}}{\text{total number of nuclei counted}} \right) \times 100$

Isolation of nuclear protein:

Reagents:

1. 5 M NaCl

   146.1 g NaCl (Sigma Chemical Co. S3014)

   Brought final volume to 500 ml with autoclaved H_2O
2. 1 M Hepes-KOH, pH 7.9

   23.8 g Hepes (Sigma Chemical Co. H0891)

   Added 80 ml autoclaved water and brought pH to 7.9 with KOH pellets. Final volume was 100 ml
3. 0.5 M EDTA, pH 8.0

   18.61 g EDTA (Sigma Chemical Co. E5134)

   Add 80 ml autoclaved water and bring pH to 8.0.

   Brought final volume to 100 ml
4. 1 M $MgCl_2$

   20.33 g $MgCl_2$ (Sigma Chemical Co. M2670)

   Brought final volume to 100 ml with autoclaved H_2O
5. IGEPAL CA-630 (Sigma Chemical Co. I3021)
6. Glycerol (Sigma Chemical Co. G5516)
7. 0.1 M Phenylmethylsulfonyl fluoride (PMSF)

   0.0174 g PMSF (Boehringer Mannheim 837 091)

   1 ml 100% ethanol and keep in freezer.
8. 0.1 M Dithiothreitol (DTT)

   0.0154 g of DTT (Bio-Rad 161-0611)

   1 ml autoclaved water
9. 0.1 M Benzamidine

   0.0156 g Benzamidine (Sigma Chemical Co. B6506)

   1 ml autoclaved water
10. 1.0 mg/ml Aprotinin

    1 mg aprotinin (Sigma Chemical Co. A1153)

    1 ml autoclaved water (aliquot and stored in freezer)
11. 1.0 mg/ml Leupeptin

    1 mg leupeptin (Sigma Chemical Co. L2884)

1 ml autoclaved water (aliquot and stored in freezer)

12. 1.0 mg/ml Pepstatin A

    1 mg pepstatin A (Sigma Chemical Co. P4265)

    Dissolved in the 100 µl DMSO (Sigma Chemical Co. D8779) and brought the volume to 1 ml with autoclaved water (aliquot and stored in freezer)

Buffers:

1. Buffer A: about 5 ml for 0.3 g tissue

Stock	Amount added	Final Conc.
IGEPAL CA-630	0.18 ml	0.6%
5M NaCl	0.9 ml	150 mM
1 M Hepes, pH 7.9	0.3 ml	10 mM
0.5 M EDTA	60 µl	1 mM
0.1 M PMSF (added last)	150 µl	0.5 mM
Sterilized water	28.41 ml	
TOTAL	30 ml	

2. Buffer C: 120 µl/0.3 g tissue

Stock	Amount added	Final Conc.
100% Glycerol	0.25 ml	25% Glycerol
1 M Hepes, KOH, pH 7.9	20 µl	20 mM
5 M NaCl	84 µl	420 mM
1 M $MgCl_2$	1.2 µl	1.2 mM
0.05 M EDTA	4 µl	0.2 mM
0.1 M PMSF	5 µl	0.5 mM
0.1 M DTT	5 µl	0.5 mM
0.1 M Benzamidine	20 µl	2 mM
1 mg/ml Aprotinin	5 µl	5 µg/ml
1 mg/ml Leupeptin	5 µl	5 µg/ml
1 mg/ml Pepstatin	5 µl	5 µg/ml

Sterilized water	595.8 µl
TOTAL	1000 µl

Procedure:

1. Buffer A was prepared and kept on ice.
2. The liver was removed from freezer and kept on dry ice. The tissue was broken into smaller pieces.
3. Approximately 0.3 g of tissue and 5 ml of cold Buffer A were added to a 15-ml Dounce tissue homogenizer. Eight strokes were made and the homogenate was transferred to a 15 ml tube.
4. The homogenate was centrifuged at 270 x g for 30 seconds to rid unbroken tissue (1000 rpm for Mistral 3000i centrifuge).
5. The supernatant was transferred to a 15 ml tube and kept on ice for at least 5 minutes.
6. The supernatant was centrifuged at 2980 x g for 20 minutes (3600 rpm for Mistral 3000i centrifuge).
7. During the 20 minute spin, buffer C was prepared.
8. The supernatant was discarded. The walls of the tube were cleaned with a Kim-wipe or cotton swab in other to rid the fat that clings to the walls.
9. The pellet was resuspended in 1 ml of buffer A and transferred to a microcentrifuge tube.
10. The resuspended pellet was centrifuged at 7,000 rpm for 3 minutes.
11. The supernatant was discarded, and the pellet was resuspended with buffer C (120 µl for 0.3 g of liver tissue). The pellet was incubated with buffer C on ice for 1 hour for high-salt extraction.
12. The tubes were spun at maximum speed for 10 minutes in microcentrifuge. The supernatant was the nuclear extract.
13. The nuclear extract was aliquoted and stored at –80 °C. One aliquot was diluted in PBS to measure the protein concentration using the BCA method (Pierce). Care was taken to avoid freeze-thawing of the nuclear extracts.

Protein assay:

***Principle**:* Cu^{+2} is reduced to Cu^{+1} by proteins (sample) in an alkaline environment (Reagent A). The chelation of two molecules of bicinchoninic acid BCA (in Reagent B) with one Cu^{+1} forms a complex, which exhibits an absorbance at 562 nm.

Reagents:

1. Reagent A (Pierce Chemical Company 23223)
2. Reagent B (Pierce Chemical Company 23224)
3. Standards - Bovine gamma globulin (Bio-Rad 500-0005) was dissolved in PBS to 1.4 mg/ml, aliquoted, and stored at -20°C. One aliquot of the 1.4 mg/ml stock solution was used to make the working standard concentrations: 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml.

Procedure:

1. 200 µl of mixed reagent (1 part Reagent A and 50 parts Reagent B) was added to each well of a 96-well plate.
2. 10 µl of each diluted sample and 10 µl of each standard was added to each well in duplicate. Note: all dilutions were made in phosphate buffered saline (PBS).
3. The 96-well plate was incubated for 30 minutes at 37°C.
4. The plate was cooled to room temperature and read at 562 nm with a Bio-Tek plate reader (Model 312, Bio-Tek Instruments, Inc.). Using the KinetiCalc software (Bio-Tek Instruments, Inc.), the concentration of protein (mg/ml) was calculated based on the standard curve.

<u>Electrophoretic mobility shift assay (EMSA)</u>:

Reagents:

1. [r-^{32}P] ATP - 6000Ci/mmol at 10mCi/ml (NEN Life Science BLU/NEG/002Z)
2. 0.5 M EDTA, pH 8.0
3. TE buffer (10 mM Tris-HCl, pH 7.5 and 1 mM EDTA)
4. T4 Polynucleotide Kinase (New England Biolabs M0201S)
5. 30% Acrylamide (29:1) (Bio-Rad Laboratories 161-0156)
6. 10% Ammonium Persulfate (APS)
   0.5 g APS (Sigma Chemical Co. A9164)
   5 ml autoclaved water

7. TEMED (Sigma Chemical Co. T7024)
8. Nonidet P-40 (Sigma Chemical Co. P4504)
9. 1 M Hepes-KOH, pH 7.9
10. 1 M KCl
11. 0.1 M Dithiothreitol (DTT, Bio-Rad 161-0611)
12. 100% Glycerol (Sigma Chemical Co. G5516)
13. NF-κB consensus oligonucleotide (Promega E329A)
14. AP-1 consensus oligonucleotide (Santa Cruz sc-2501)
15. 1 mg/ml Poly (dI-dC)·(dI-dC) (Amersham Pharmacia Biotech 277880)
    Dissolved in TE (10 mM Tris and 1 mM EDTA, pH 7.5)
16. 10x TBE
    108 g Tris base (Boehringer Mannheim 1814 273)
    54 g Boric acid (Gibco 15583-024)
    40 ml 0.5 M EDTA, pH 8.0 (bring to 1 liter with water)

Procedure:

1. Radiolabel NF-κB and AP-1 probe
   a. Add below into an microcentrifuge tube

Nuclease-free water	5 µl
NF-κB or AP-1 consensus oligonucleotide (1.75 pmol/µl)	2 µl
T4 Polynucleotide Kinase 10x Buffer	1 µl
[r-^{32}P] ATP	1 µl
T4 Polynucleotide Kinase (10 U/µl)	1 µl

   a. The reactions were incubated at 37 °C for 20 minutes.
   b. Each reaction was stopped with 1 µl of 0.5 M EDTA with phenol red.
2. Purification of labeled probe
   The labeled oligonucleotide was separated from unincorporated nucleotides by passing the reactions through a Bio-gel column (Bio-Rad Laboratories 151-0440). The components of each reaction were added to the center of the column and washed with TE buffer. The radioactive peak was collected, and 1 µl was counted using a liquid scintillation counter. The average cpm for 1 µl was 50,000. The radiolabeled probe was stored at –20 °C until use.
3. Electrophoresis Mobility Shift Assay (EMSA)

a. Made 5x binding buffer (according to Promega):

Stock	For 5 ml	5x binding buffer (stock)	1x binding buffer (final concentration in reaction)
1 M Hepes, pH 7.9	250 µl	50 mM	10 mM
1 M KCl	1.25 ml	250 mM KCl	50 mM KCl
0.5 M EDTA	10 µl	1 mM EDTA	0.2 mM EDTA
0.1 M DTT	625 µl	12.5 mM DTT	2.5 mM DTT
100% glycerol	2.5 ml	50% glycerol	10% glycerol
NP-40	12.5 µl	0.25% NP-40	0.05% NP-40
Autoclaved H_2O	352.5 µl		

b. A 7% polyacrylamide gel was cast

	30 ml
Water	21.19 ml
30% acrylamide/bis(29:1)	7 ml
TBE (10x)	1.5 ml
10% APS	0.3 ml
TEMED	15 µl

c. The gel was allowed to solidify for at least 45 minutes.

d. The reaction was carried out as follows:

Nuclear extracts	5 µg
5x binding buffer	4 µl
Poly (dI-dC)	0.2 µg
Radiolabeled probe	2 µl (100,000 cpm)
Autoclaved water	brought total volume to 20 µl

All contents except for the radiolabeled probe were pre-incubated on ice for 5 minutes. The radiolabeled NF-κB or AP-1 probe was added to the reaction and incubated for 15 minutes at room temperature.

e. The comb was removed and the wells were rinsed with 0.5 X TBE buffer using a needle and syringe. The 20 µl reaction mixture was added to each well of the 7%

polyacrylamide gel and electrophoresed for 2 hours at 180 volts using 0.5 x TBE as the running buffer. A positive control was included with each gel (HeLa cell extract, Santa Cruz).

f. In order to confirm binding specificity of NF-κB, 1 μg of antibody raised against p50 or p65 were added to the reaction before adding the NF-κB probe (during the 10 minute pre-incubation).

After removing the gel from the running apparatus, the gel was attached to filter paper and covered with plastic wrap. The gel was dried under vacuum and exposed overnight at –80 °C to Kodak XOMAT-AR film. The radioactivity was counted with a radioanalytic imaging system (Ambis, San Diego, CA).

Statistical analysis:

Results were first analyzed by one-way analysis of variance and Dunnett's post hoc test. The results were reported as means ± standard error of mean (SEM).

Results

In this study, we have examined the hypothesis that specific PCB congeners could activate oxidative stress-related transcription factors and induce cell proliferation. In the first study, rats received corn oil, PCB-77 (30 or 150 μmol/kg), PCB-153 (30 or 150 μmol/kg), or combination of the two PCBs (30 μmol/kg for each PCB). Liver tissue was collected at 2, 6, 24 hours or 2, 6 and 10 days after the injection. The liver/body weight ratio was not significantly different among any of those groups before day 6 (Table 2.1). The high dose of PCB-77 significantly increased the liver to body weight ratio at day 6, and by day 10 the ratio was significantly increased by both doses of PCB-77, the high dose of PCB-153 and the combination of two PCBs. The DNA binding activities of the transcription factors NF-κB and AP-1 were examined by EMSA, and were not significantly changed by any of the PCBs at any time points, although there was a slight increase in the DNA binding activities of NF-κB by high dose of PCB-153 and the combination of both PCBs after 2 days (Tables 2.2 and 2.3). The specificity of the DNA binding of two factors was determined by supershift using nuclear extract from high-dose PCB-153 group and antibodies against NF-κB subunits (p50 and p65) or AP-1 subunits

(c-*Fos* and c-*Jun*). The presence of either the anti-p65 or anti-p50 antibody resulted in a supershifted complex, indicating that both p50 and p65 subunits were present in the NF-κB protein-DNA complex (Figure 2.1). Antibody specific to c-*Fos* led to a supershifted complex and the anti-c-*Jun* antibody caused a 30% reduction in the intensity of the AP-1 protein-DNA complex (Figure 2.2), indicating the presence of both c-*Fos* and c-*Jun* in the AP-1 protein-DNA complex.

To measure the rate of cell proliferation, we quantified the labeling index after rats were administered BrdU. The labeling indexes of the PCB-treated groups were not significantly different from the control group at any of the six time points tested (Table 2.4).

In our initial study, there was a slight increase in the DNA binding activity of NF-κB and in the labeling index in rats treated with PCB-153 and the combination of two PCBs at day 2, but these differences were not statistically significant. Based on these observations, we administered higher doses of PCBs: rats received corn oil, PCB-77 (150 or 300 μmol/kg), PCB-153 (150 or 300 μmol/kg), or both PCBs (150 μmol/kg each), and rats were euthanized after 2 or 6 days. The liver to body weight ratio was increased in both PCB-77 groups and the group receiving both PCBs at day 6, but PCB-153 did not have a significant effect on the liver weight (Table 2.5). At day 2, there was no difference in the liver to body weight ratio between any PCB group and the control group. EMSAs were used to determine the DNA binding activity of NF-κB and AP-1. PCB-153 treatment increased NF-κB binding activity at day 2, but not at day 6; the other treatments had no effect (Figures 2.3 and 2.4). The DNA binding activity of AP-1 was not increased by any PCB at either time point (Figures 2.5 and 2.6).

The rate of cell proliferation in the second study, as measured by quantitation of the BrdU labeling index, was increased in the high-dose (300 μmol/kg) PCB-153 group and the group receiving both PCBs compared to the corn oil-treated controls at day 2 (Figure 2.7). The labeling index was not significantly different in rats treated with either dose of PCB-77, or the low dose of PCB-153, compared to corn oil-treated controls. At day 6, there were no significant differences in the BrdU labeling index between any PCB group and the control group.

Table 2.1. Effect of PCBs on liver weight

Treatment	Dose (μmol/kg)	Liver weight (% of body weight)					
		2 h	6 h	24 h	2 days	6 days	10 days
Corn Oil		4.85 ± 0.10	4.25 ± 0.10	4.83 ± 0.13	4.82 ± 0.14	4.60 ± 0.19	4.40 ± 0.05
PCB-77	30	4.82 ± 0.04	4.44 ± 0.17	4.98 ± 0.06	5.10 ± 0.15	4.93 ± 0.02	5.07 ± 0.15*
PCB-77	150	4.56 ± 0.08	4.74 ± 0.01	5.24 ± 0.10	5.25 ± 0.14	5.52 ± 0.09*	5.59 ± 0.07*
PCB-153	30	4.82 ± 0.13	4.44 ± 0.16	5.01 ± 0.19	4.77 ± 0.12	5.01 ± 0.02	4.78 ± 0.03
PCB-153	150	4.73 ± 0.03	4.24 ± 0.09	4.93 ± 0.11	4.71 ± 0.03	5.01 ± 0.23	5.13 ± 0.20*
Both PCBs	30+30	4.96 ± 0.05	4.76 ± 0.14	5.13 ± 0.18	5.02 ± 0.03	4.95 ± 0.17	5.38 ± 0.17*

Each value is the mean ± SEM. PCB-77 or PCB-153 (30, 150 μmol/kg) or both PCBs were given in corn oil as a single i.p. dose. Control animals received the vehicle. Each group contained 3 animals. *Values are significantly different from controls ($p < 0.05$).

Figure 2.1. Determination of NF-κB specific binding. Antibodies specific for NF-κB subunits (p50 and p65) were added to the reaction containing a radiolabeled NF-κB oligonucleotide and liver nuclear extracts from high level PCB-153 group.

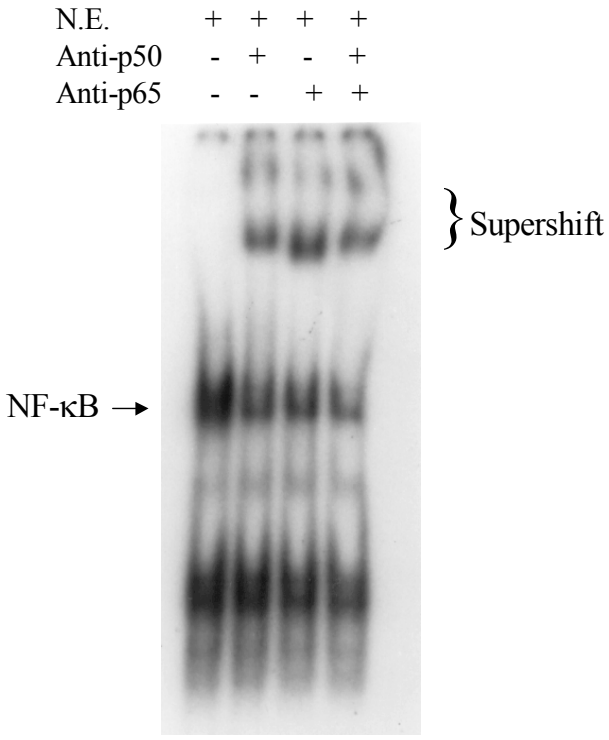


Figure 2.2. Determination of AP-1 specific binding. Antibodies specific for AP-1 subunits (c-*Fos* and c-*Jun*) were added to the reaction containing a radiolabeled AP-1 oligonucleotide and liver nuclear extracts from high level PCB-153 group.

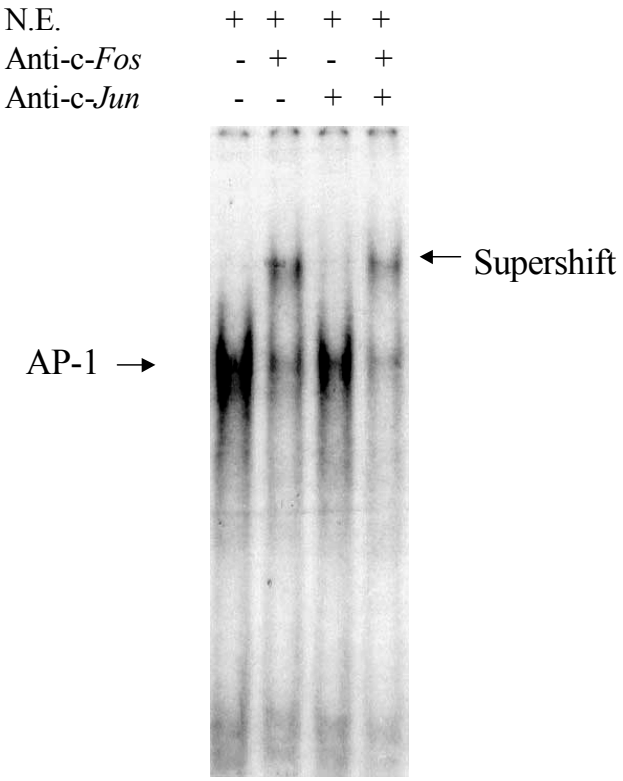


Table 2.2. Effect of PCBs on hepatic DNA binding activity of NF-κB

		Hepatic NF-κB Activity (% of control)					
Treatment	Dose (μmol/kg)	2 h	6 h	24 h	2 days	6 days	10 days
Corn Oil		100 ± 8	100 ± 9	100 ± 4	100 ± 15	100 ± 11	100 ± 2
PCB-77	30	92 ± 2	63 ± 8	96 ± 21	84 ± 09	1.81 ± 36	132 ± 19
PCB-77	150	106 ± 11	68 ± 6	119 ± 28	105 ± 27	172 ± 32	108 ± 16
PCB-153	30	122 ± 21	100 ± 24	102 ± 21	124 ± 29	118 ± 22	108 ± 17
PCB-153	150	60 ± 10	77 ± 32	85 ± 11	169 ± 34	124 ± 36	100 ± 15
Both PCBs	30+30	75 ± 19	102 ± 5	140 ± 24	232 ± 56	172 ± 35	108 ± 6

Each value is the mean ± SEM. PCB-77 or PCB-153 (30, 150 μmol/kg) or both PCBs were given in corn oil as a single i.p. dose. Control animals received the vehicle alone. Each group contained 3 animals. EMSAs were performed using a radiolabeled NF-κB oligonucleotide with rat liver nuclear extracts. NF-κB DNA binding activity was quantified by measurement of the net cpm of NF-κB band using a radioanalytic imaging system (Ambis Corp., San Diego, CA).

Table 2.3. Effect of PCBs on hepatic DNA binding activity of AP-1

Treatment	Dose (μmol/kg)	Hepatic AP-1 Activity (% of control)					
		2h	6 h	24 h	2 days	6 days	10 days
Corn Oil		100 ± 6	100 ± 9	100 ± 18	100 ± 15	100 ± 9	100 ± 11
PCB-77	30	86 ± 9	73 ± 8	108 ± 11	95 ± 9	121 ± 26	112 ± 6
PCB-77	150	106 ± 8	109 ± 5	121 ± 16	132 ± 20	92 ± 6	96 ± 12
PCB-153	30	105 ± 15	105 ± 24	102 ± 11	114 ± 24	88 ± 21	94 ± 6
PCB-153	150	90 ± 8	82 ± 25	111 ± 21	144 ± 3	73 ± 16	103 ± 16
Both PCBs	30+30	121 ± 14	133 ± 8	131 ± 12	192 ± 20	147 ± 35	116 ± 8

Each value is the mean ± SEM. PCB-77 or PCB-153 (30, 150 μmol/kg) or both PCBs were given in corn oil as a single i.p. dose. Control animals received the vehicle alone. Each group contained 3 animals. EMSAs were performed using a radiolabeled AP-1 oligonucleotide with liver nuclear extracts from each rat. AP-1 DNA binding activity was quantified by measurement of the net cpm of AP-1 band using a radioanalytic imaging system (Ambis Corp. San Diego, CA).

Table 2.4. Effect of PCBs on hepatic cell proliferation

Treatment	Dose (μmol/kg)	BrdU index (100%) 2 h	6 h	24 h	2 days	6 days	10 days
Corn Oil		0.93 ± 0.31	0.81 ± 0.14	1.15 ± 0.35	0.87 ± 0.28	1.04 ± 0.26	1.08 ± 0.20
PCB-77	30	2.23 ± 1.33	0.60 ± 0.07	1.01 ± 0.15	0.97 ± 0.26	1.18 ± 0.36	0.86 ± 0.34
PCB-77	150	0.68 ± 0.16	0.55 ± 0.11	1.02 ± 0.25	0.67 ± 0.05	0.80 ± 0.18	1.14 ± 0.38
PCB-153	30	2.02 ± 0.53	0.80 ± 0.10	0.60 ± 0.31	1.10 ± 0.53	1.00 ± 0.27	1.22 ± 0.25
PCB-153	150	1.09 ± 0.04	0.66 ± 0.08	0.81 ± 0.27	1.62 ± 0.43	0.71 ± 0.24	1.41 ± 0.26
Both PCBs	30+30	1.39 ± 1.17	0.63 ± 0.01	0.91 ± 0.26	2.09 ± 0.35	0.76 ± 0.07	0.85 ± 0.36

Each value is the mean ± SEM. PCB-77 or PCB-153 (30, 150 μmol/kg) or both PCBs were given in corn oil as a single i.p. dose. Control animals received the vehicle alone. Each group contained 3 animals. Rats were administrated BrdU either by a 2-day infusion using Alzet osmotic pumps (10 days only) or by a s.c injection (all other time points). Tissue sections were immunohistochemically stained for BrdU and labeling indexes were determined.

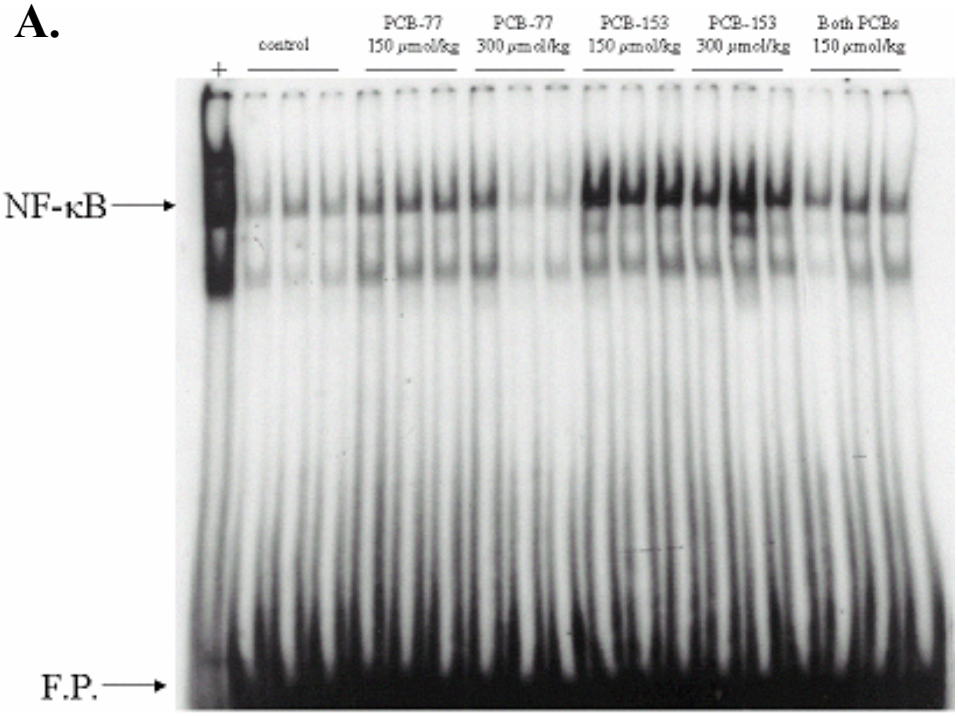
Table 2.5. Effect of high dose of PCBs on liver weight

Treatment	Dose (μmol/kg)	Liver weight (% of body weight) 2 days	6 days
Corn Oil		5.20 ± 0.08	4.86 ± 0.14
PCB-77	150	5.50 ± 0.10	5.90 ± 0.27*
PCB-77	300	5.60 ± 0.13	5.84 ± 0.25*
PCB-153	150	5.18 ± 0.15	5.19 ± 0.15
PCB-153	300	5.23 ± 0.21	5.52 ± 0.09
Both PCBs	150+150	5.79 ± 0.12	6.31 ± 0.19*

Each value is the mean ± SEM. PCB-77 or PCB-153 (150, 300 μmol/kg) or both PCBs were given in corn oil as a single i.p. dose. Control animals received the vehicle alone. Each group contained 5 animals. *Values are significantly different from controls ($p < 0.05$).

Figure 2.3. Effect of high dose of PCBs on the hepatic DNA binding activity of NF-κB. EMSAs were performed using a radiolabeled NF-κB oligonucleotide with control HeLa nuclear extract or liver nuclear extracts from individual rats. Lane 1, HeLa cell nuclear extract as a positive control; remaining lines, liver nuclear extracts from a single rat (5 μg). F.P., free probe. A. EMSA in 2-day study. B. EMSA in 6-day study.

A.


control
PCB-77 150 μmol/kg
PCB-77 300 μmol/kg
PCB-153 150 μmol/kg
PCB-153 300 μmol/kg
Both PCBs 150 μmol/kg
+
NF-κB
F.P.

B.

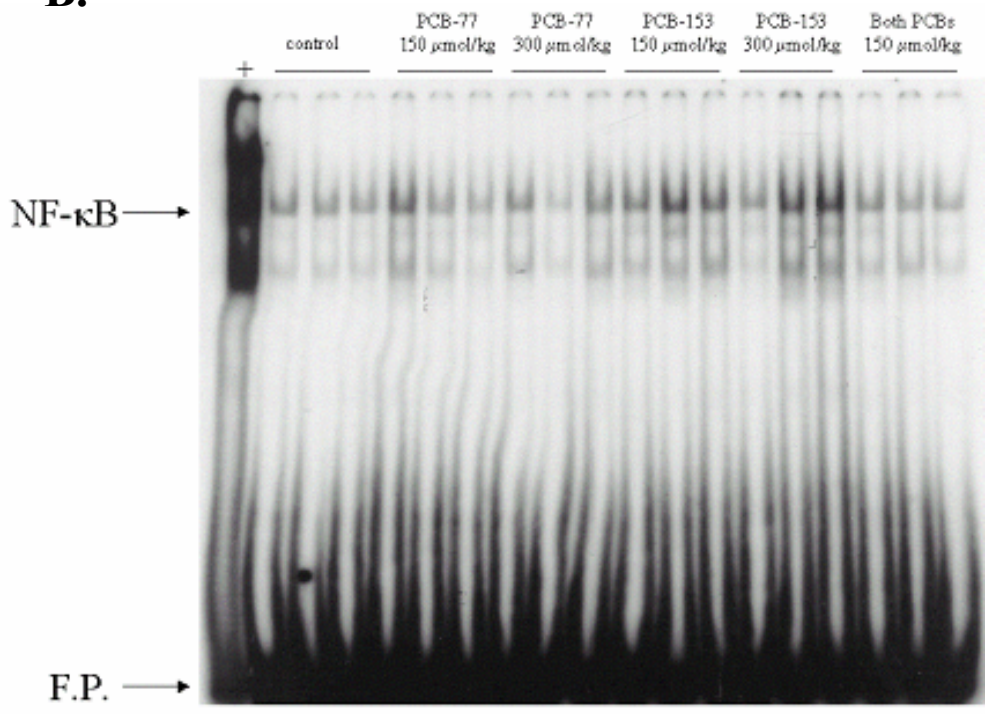

control
PCB-77 150 μmol/kg
PCB-77 300 μmol/kg
PCB-153 150 μmol/kg
PCB-153 300 μmol/kg
Both PCBs 150 μmol/kg
+
NF-κB
F.P.

Figure 2.4. Relative radioactive counts of NF-κB bands from the EMSA data (Figure 2.3). Quantitation of the specific NF-κB band was determined by subtracting background counts from the count in each NF-κB band. The radioactive counts of NF-κB from control groups were set as 100. *Values are significantly different from control group ($p < 0.05$).

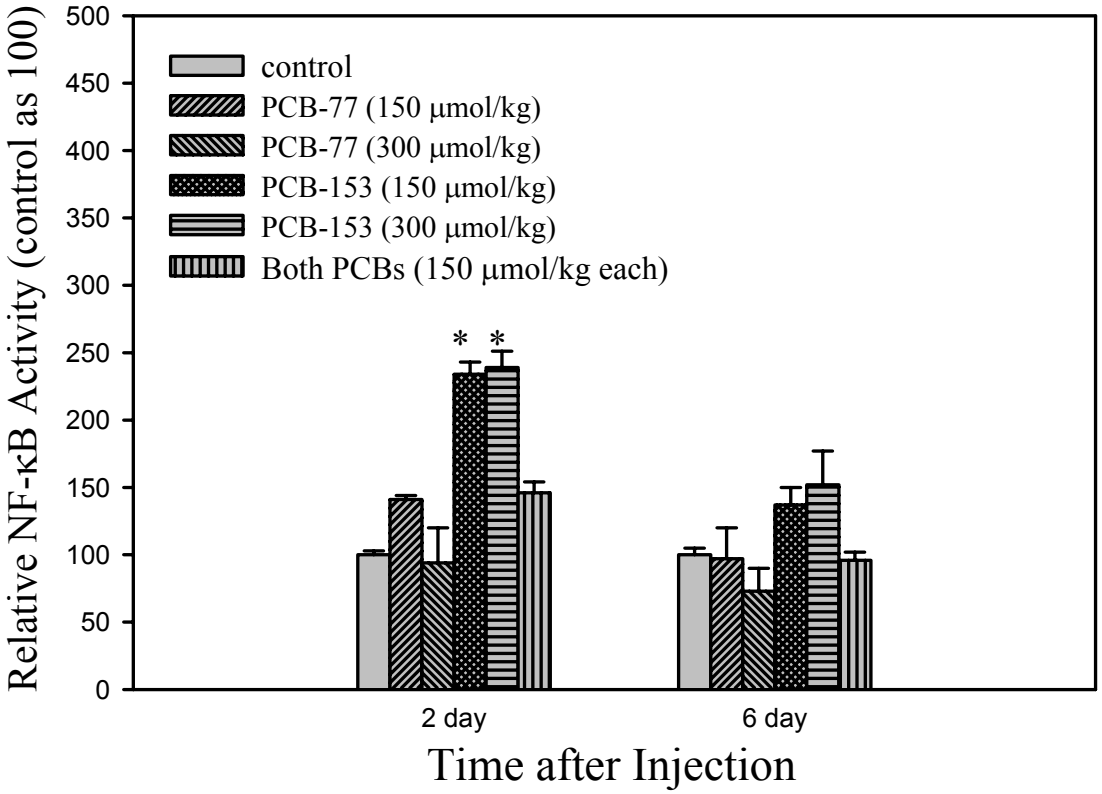
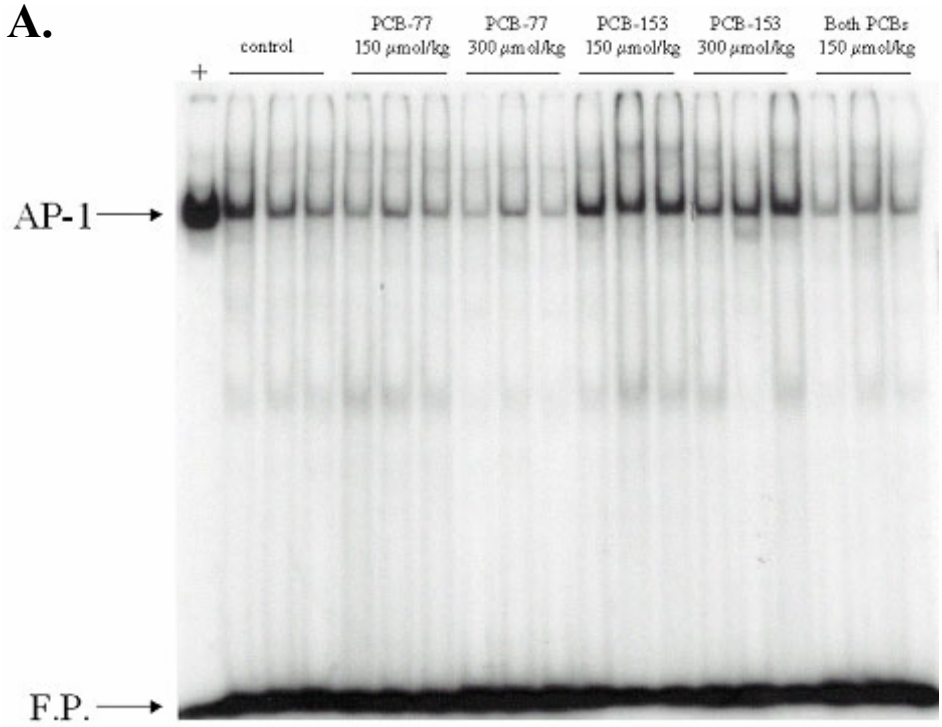

Relative NF-κB Activity (control as 100)
500
450
400
350
300
250
200
150
100
50
0
control
PCB-77 (150 μmol/kg)
PCB-77 (300 μmol/kg)
PCB-153 (150 μmol/kg)
PCB-153 (300 μmol/kg)
Both PCBs (150 μmol/kg each)
* *
2 day
6 day
Time after Injection

Figure 2.5. Effect of high dose of PCBs on the hepatic DNA binding activity of AP-1. EMSA were performed using a radiolabeled AP-1 oligonucleotide with control HeLa nuclear extract or liver nuclear extracts from individual rats. Each line contains extracts from a single rat. Lane 1, HeLa cell nuclear extract as a positive control; remaining lanes, liver nuclear extracts from a single rat (5 μg). F.P., free probe. A. EMSA in 2-day study. B. EMSA in 6-day study.

A.


control
PCB-77 150 μmol/kg
PCB-77 300 μmol/kg
PCB-153 150 μmol/kg
PCB-153 300 μmol/kg
Both PCBs 150 μmol/kg
+
AP-1
F.P.

B.

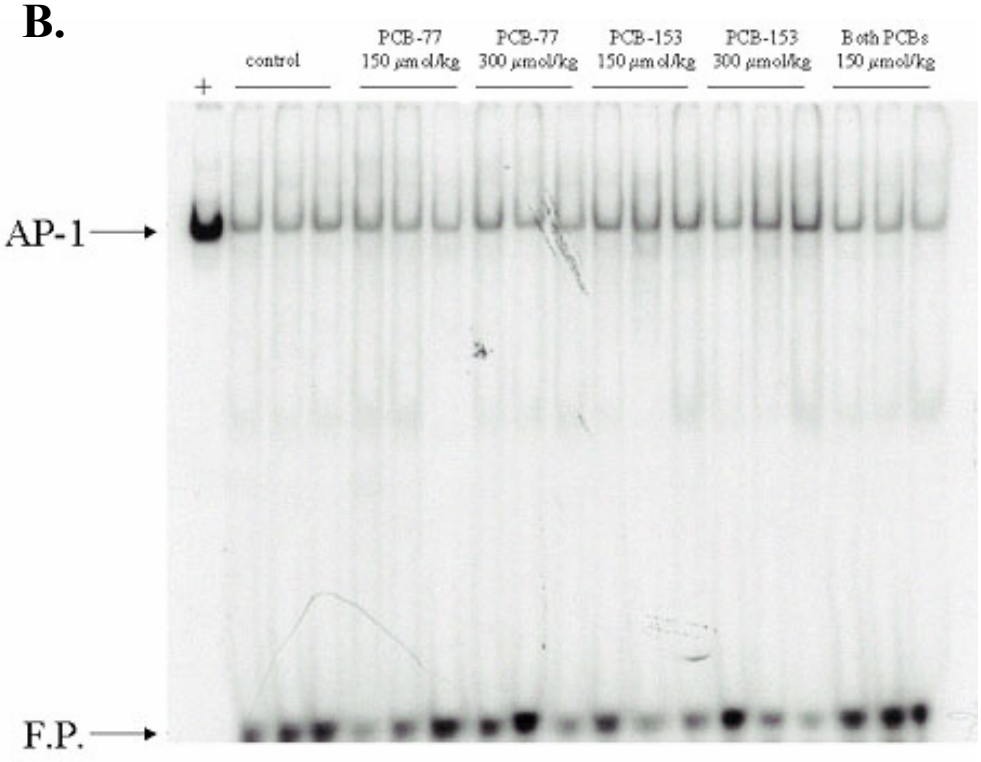

control
PCB-77 150 μmol/kg
PCB-77 300 μmol/kg
PCB-153 150 μmol/kg
PCB-153 300 μmol/kg
Both PCBs 150 μmol/kg
+
AP-1
F.P.

Figure 2.6. Effect of high dose of PCBs on the hepatic DNA binding activity of AP-1. Relative radioactive counts of AP-1 bands from the EMSA data (Figure 2.5). Quantitation of the specific AP-1 band was determined by subtracting background counts from the count in each AP-1 band. The radioactive counts of AP-1 bands from control groups were set as 100.

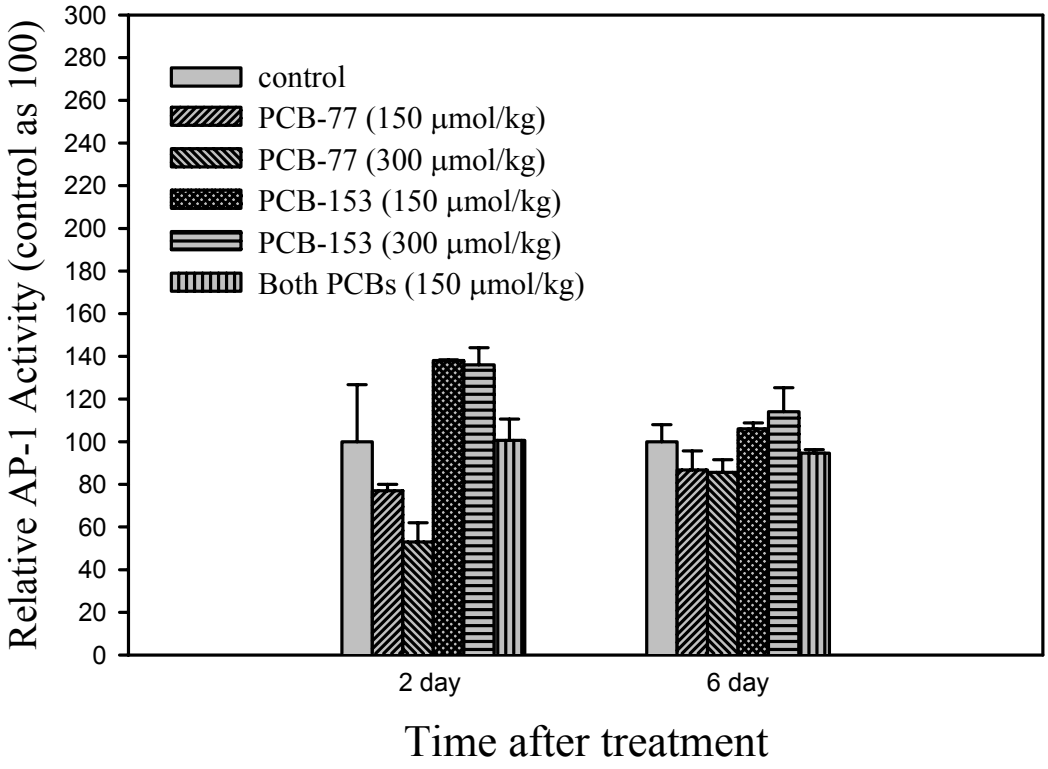
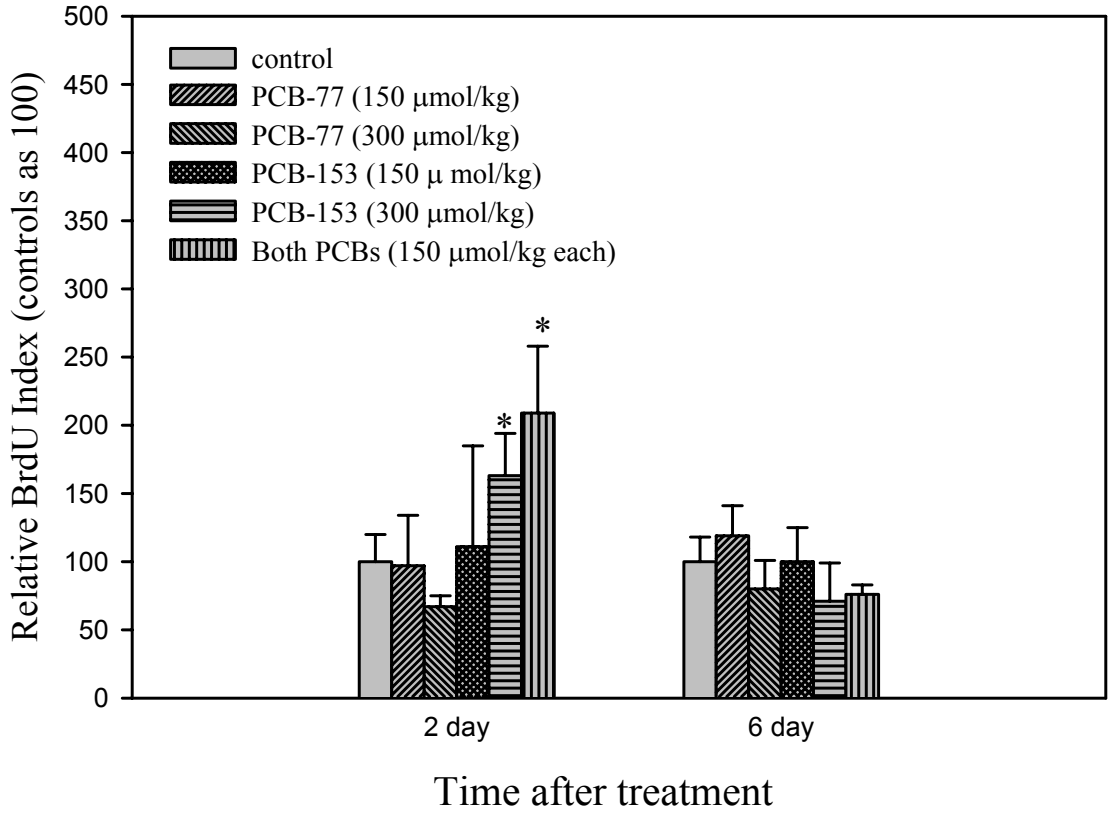

Relative AP-1 Activity (control as 100)
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
control
PCB-77 (150 μmol/kg)
PCB-77 (300 μmol/kg)
PCB-153 (150 μmol/kg)
PCB-153 (300 μmol/kg)
Both PCBs (150 μmol/kg)
2 day
6 day
Time after treatment

Figure 2.7. Effect of high dose of PCBs on cell proliferation. Rats were s.c. injected with BrdU 2 hours before euthanasia. Tissue sections were immunohistochemically stained for BrdU and labeling indexes were determined. Values of control groups are set as 100. Each group contained 5 animals. *Values are significantly different from control groups ($p < 0.05$).


500
450
400
350
300
250
200
150
100
50
0
Relative BrdU Index (controls as 100)
control
PCB-77 (150 μmol/kg)
PCB-77 (300 μmol/kg)
PCB-153 (150 μ mol/kg)
PCB-153 (300 μmol/kg)
Both PCBs (150 μmol/kg each)
*
*
2 day
6 day
Time after treatment

Discussion

In this study, we have examined the hypothesis that single dose of specific PCB congeners could activate oxidative stress-sensitive transcription factors, namely NF-κB and AP-1. We found that PCB-153 at the highest dose tested transiently activated hepatic NF-κB in rats. This NF-κB activation by PCB-153 could be explained as results of oxidative stress caused by PCB-153, or the interactions between glucocorticoid receptor and PCB-153. PCB-153, a typical non-coplanar PCB congener, induces cytochrome P450 2B family. Reactive oxygen species, such as superoxide and H_2O_2, can leak out of cytochrome P450 (Bondy & Naderi 1994; Goeptar *et al*. 1995), which would contribute to NF-κB activation. Another possible mechanism of NF-κB activation by PCB-153 is that PCB-153 or its metabolites could directly act on glucocorticoid receptor (GR), which, when activated, inhibits the activity of NF-κB (De Bosscher *et al*. 1997; De Bosscher *et al*. 2000; Heck *et al*. 1997), by direct or indirect mechanisms. Glucocorticoids induce transcription and protein synthesis of IκB (Auphan *et al*. 1995; Scheinman *et al*. 1995). Activated GR also antagonizes NF-κB activity through direct protein-protein interaction, and either prevents NF-κB binding to DNA (Adcock *et al*. 1995; Steer *et al*. 2000) or associates with NF-κB bound to κB-binding site (De Bosscher *et al*. 2000; Nissen & Yamamoto 2000). Also, activated GR competes with NF-κB for nuclear coactivators (McKay & Cidlowski 2000; Sheppard *et al*. 1998). PCBs metabolites have been shown to act as competitive antagonist at the GR (Johansson *et al*. 1998). In primary rat hepatocytes, PCB-153 and phenobarbital have been shown to suppress the expression of two sulfotransferase genes that are positively regulated by glucocorticoids (Runge-Morris 1998), which suggested a counteracting effect between PCB-153 and glucocorticoids although it is no clear if GR was involved.

Our results indicated that PCB-77 alone, or PCB-77 and PCB-153 together, could not activate NF-κB. One mechanism by which coplanar PCBs could influence NF-κB activation is through their induction of the Aryl hydrocarbon (Ah) receptor. The activated Ah receptor has been found to bind to the p65 subunit of NF-κB (Tian *et al*. 1999), and this binding caused mutual function repression. TCDD has been found to activate NF-κB in wild-type mouse hepatoma Hepa-1 cells, but this increase in NF-κB DNA binding

activity was mainly due to an increase in p50/p50 complexes (Puga *et al*. 2000). Our lab has recently shown that NF-κB was activated after 4 injections of PCB-77 in an initiation-promotion study (Tharappel *et al*. 2002), which implied that this inhibition could be overcome by long-term treatment.

Our study did not show any significant increase in hepatic DNA binding activity of AP-1, although there was a slightly increase by PCB-153 on day 2 (Figure 2.5). AP-1, as another oxidative stress-sensitive transcription factor, has been shown to be activated in rats with much higher dose of PCBs, in which 4 injections of PCB-3, PCB-28, or PCB-153 (Oakley *et al*. 2001), 6 injections of PCB-77 or PCB-153 (Twaroski *et al*. 2001a), and 4 injections of PCB-77, PCB-153 or both PCBs (Tharappel *et al*. 2002) were given to rats. Recently, the IKK/NF-κB pathway has been shown to negatively modulate TNF-α-mediated c-Jun N-terminal protein kinase (JNK) activation (De Smaele *et al*. 2001; Javelaud & Besancon 2001; Tang *et al*. 2001), and c-Jun/AP-1 activation was inhibited by NF-κB in rat hepatocyte cell line RALA255-10G (Liu *et al*. 2002). This negative crosstalk between NF-κB and JNK/c-Jun/AP-1 has been suggested to be mediated partly through the NF-κB-induced X-chromosome–linked inhibitor of apoptosis (XIAP) (Tang *et al*. 2001). Whether there is an interaction between PCBs-mediated NF-κB activation and c-Jun/AP-1 pathway is unclear, but studies with higher doses of PCBs showed that oxidative stress caused by higher dose of PCBs could induce the AP-1 activation.

We have shown that high dose of PCB-153 and both PCBs induced cell proliferation (Figure 2.6). The commercial PCBs mixture Aroclor 1254 has been shown to induce hepatocyte proliferation in rats (Whysner & Wang 2001) and mice (Madra *et al*. 1995). The induction of cell proliferation correlated somewhat with increased NF-κB DNA binding activity, although this correlation was not absolute. This suggests that NF-κB activation alone is not sufficient to cause increased cell proliferation after PCB exposure.

Whether synergism exists between PCB congeners has attracted the attention of many investigators. We did not observe a synergistic effect between PCB-77 and PCB-153 on NF-κB activation, which is in agreement with a previous study (Tharappel *et al*. 2002). In contrast, cell proliferation was significantly induced by the combination of the two PCBs (150 μmol/kg each), while it was not significantly changed by either one of the

PCBs at the same concentration. Tharappel *et al*. observed no synergism between PCB-77 and PCB-153 on cell proliferation and focal growth during tumor promotion (Tharappel *et al*. 2002). In other studies, antagonism has been shown between coplanar PCB congeners (77 or 126) and non-coplanar PCB congener (153) during tumor promotion (Berberian *et al*. 1995; Haag-Gronlund *et al*. 1998). How the PCB congeners exert synergistic effect on cell proliferation in early responses is not clear, but accumulating data suggested that depletion of hepatic retinol content by PCB-77 could contribute to the cell proliferation. Retinoic acid has been shown to inhibit DNA synthesis in primary rat hepatocytes (Ikeda & Fujiwara 1993). In rat liver, restoration of retinoic acid by dietary supplements inhibited ethanol-induced hepatocyte proliferation (Chung *et al*. 2001), which suggested that depletion of retinol content in liver contributes to cell proliferation. Both PCB-153 and PCB-77 lowered retinyl palmitate in liver, with a more pronounced effect by PCB-77, and the combination of these two PCBs produced a synergetic effect (Berberian *et al*. 1995). Durham and Brouwer have shown that hepatic retinol content was decreased (34% of control) 3 days after a single dose of PCB-77 (Durham & Brouwer 1989). Thus, depletion of hepatic retinol by PCB-77 could contribute to the cell proliferation by PCB-153, and could be a possible mechanism of the synergism.

In the rats treated with highest dose of PCB-153, increased cell proliferation and NF-κB activation occurred in the same animals. Whether there is a relationship between NF-κB activation and cell proliferation following PCB-153 treatment need to be further investigated. The role of NF-κB in cell growth and tumorigenesis has been studied extensively due to the ability of NF-κB to modulate the transcription of genes whose products are involved in cell proliferation and the suppression of apoptotic cascades (Mayo & Baldwin 2000). Changes in those gene products are important for us to understand the possible connections between NF-κB activation and cell proliferative responses during tumor formation by PCBs.

Chapter 3. Effect of PCB-153 on hepatocyte proliferation and apoptosis in mice deficient in the p50 subunit of NF-κB

Introduction

Polychlorinated biphenyls (PCBs) are a group of 209 synthetic aromatic isomers that are different in the position and number of chloride atoms in the biphenyl structure. Because of their physical properties, chemical stability, inflammability, and solubility in organic solvents, the use of PCBs was once widespread in industries until the late 1970s. Due to their stability and lipophilicity, they are widely distributed in the environment and bioaccumulate and bioconcentrate in the food chain, thus posing a health hazard to animals and humans (Safe *et al*. 1985; Safe 1994). Toxicity studies have shown that PCB mixtures can cause cancer in experimental animals, and that PCBs have tumor promoting activity (Glauert 2001; Silberhorn *et al*. 1990).

PCBs can be divided into two groups according to the different position of chlorination (Safe 1994; Silberhorn *et al*. 1990). Coplanar PCB congeners substituted in both *para* and at least two *meta* positions but not in any of the *ortho* positions may form a coplanar configuration similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and bind strongly to Ah receptor (AhR); the binding to Ah receptor induces expression of AhR-regulated genes including cytochrome P450 1A1 and 1A2 (CYP 1A1, 1A2). On the other hand, PCB congeners with chlorine substitutes in two *ortho*-positions are non-coplanar, which have low affinity to Ah receptor but induce a battery of drug-metabolizing enzymes including cytochrome P450 2B1 and 2B2 (CYP2B1, CYP2B2), in a similar pattern as phenobarbital.

PCBs promote the development of cancer in rodent liver when the animals pretreated with a genotoxic (initiating) carcinogen. The exact mechanisms by which PCBs exert their promoting activities are still unknown. Mechanisms including the inhibition of apoptosis and induction of cell proliferation in preneoplastic cells have been hypothesized. Recently, hepatic tumor promoters such as peroxisome proliferators and phenobarbital have been found to activate the transcription factor NF-κB, which regulates the expression of cell proliferation- and apoptosis-related genes.

In hepatocytes, the major form of NF-κB consists of two subunits, a 50-kDa (p50) and a 65-kDa (p65) polypeptide that are complexed to an inhibitory subunit (IκB) in the cytoplasm. Upon stimulation, IκB is phosphorylated, ubiquinated and degraded, allowing translocation of NF-κB into the nucleus. Among the NF-κB-regulated genes, cyclin D1 controls the cell cycle checkpoint at G1/S phase (Guttridge *et al*. 1999; Hinz *et al*. 1999), and NF-κB is required for the initiation of cyclin D1 transcription and hyperphosphorylation of pRB, which lead cells to progress through G1 and entry into S phase (Biswas *et al*. 2000; Henry *et al*. 2000; Joyce *et al*. 1999; Kaltschmidt *et al*. 1999). NF-κB also regulates expression of certain anti-apoptotic genes, such as cIAPs (Wang *et al*. 1998) and Bcl-2/Bcl-x_L (Lee *et al*. 1999; Tamatani *et al*. 1999; Wang *et al*. 1999). Knockout studies revealed that p65 subunit of NF-κB is essential for cell to survive the TNF-α induced apoptosis, because deficiency in p65 results in embryonic lethality (Beg *et al*. 1995). Inhibition of NF-κB by superrepressor IκBα that has mutated phosphorylation sites preventing its degradation, results in extensive hepatocyte apoptosis after partial hepatectomy. Although p65 null mice die during embryogenesis, p50-/- mice are viable, thus providing a model to study the role of p65/p50 dimer in the regulation of cell proliferation and cell apoptotic death.

Our previous studies have indicated that 2,2',4,4',5,5'-hexachlorobiphenyl (PCB-153), a non-coplanar PCB congener, could activate hepatic NF-κB after a single dose (Chapter 2) or during the promotion stage of hepatocarcinogenesis (Tharappel *et al*. 2002). In chapter 2, hepatocyte proliferation was induced by PCB-153, which correlated the hepatic NF-κB activation in the same animals. Thus, the purpose of this study was to determine the role of NF-κB in PCB-induced cell proliferation and the specific proteins involved in the pathway. Also, the effects on the apoptotic cell death after treatment with non-coplanar PCB in these p50-/- mice were studied.

Materials and Methods

Materials: 2,2',4,4',5,5'-hexachlorobiphenyl (PCB-153) were synthesized and characterized as described previously (Schramm *et al*. 1985). Vitamin E stripped corn oil was obtained from Acros Organics (Morris Plains, NJ). Alzet osmotic pumps (model

2ML1) were obtained from Alza Scientific Products, Palo Alto, CA. The anti 5-bromo-2'-deoxyuridine (BrdU) antibody was purchased from Becton-Dickinson (San Jose, CA). The Antigen Retrieval Citra solution was purchased from BioGenex (San Ramon, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Experimental design and animal treatment: Eight weeks old male F_2 (B6.129) mice homozygous for NF-κB (p50) mutation and wild type controls were obtained from our breeding colony (originally purchased from the Jackson Laboratory, Bar Harbor, ME). In the 2-day study, 15 transgenic mice and 15 wild type littermates received a single i.p. injection of either corn oil or PCB-153 (300 μmol/kg) and were euthanized 2 days later. All mice received an s.c. injection of BrdU (100 mg/kg) 2 hours before euthanasia. In the 21-day study, 10 transgenic mice and 18 wild type mice received six i.p. injections of corn oil or PCB-153 (100 μmol/kg) and were euthanized four days after the last injection. Three days before being euthanized, all mice were surgically implanted s.c. with an osmotic pump (Alza Scientific Products, Palo Alto, CA, model 1003D) containing BrdU (20mg/ml, 10 μl/hour). Livers were removed from each mouse and pieces from each liver were fixed in 10% formalin and processed for histology and the remaining liver was snap frozen in liquid N_2 and stored in –80°C.

Nuclear extract preparation:

Nuclear protein was preparation as described in chapter 2.

Electrophoresis mobility shift assay (EMSA):

The NF-κB DNA binding activity was measured as described in chapter 2.

BrdU immunohistochemical staining:

As described in chapter 2.

***In situ* cell death detection assay (TUNEL):**

Reagents:

1. Hemo-de (Scientific Safety Solvent 15-182-507A)
2. Ethanol
3. 10 mM Tris-HCl, pH 7.4-8.0
4. PBS - as described in chapter 2
5. Proteinase K
   20 μg/ml, dissolved in 10 mM Tris-HCl, pH 7.4-8.0
6. H_2O_2 (Sigma Chemical Co. H0904)
7. Methyl Green (Vector, H3402)
8. *In situ* cell death detection assay kit (Intergen S7101)

Procedure:

1. Paraffin slides were incubated in 60 °C oven for 30 minutes.
2. The slides were washed in Hemo-De 3 times, 5 minutes each time.
3. The slides were rehydrated through graded series of ethanol (100%, 100%, 95%) and water.
4. The slides were incubated with proteinase K (20 μg/ml in 10 mM Tris/HCl, pH7.4-8.0), at 37 °C for 15 minutes.
5. The slides were rinsed with PBS for 2 times, 2 minutes each time.
6. The slides were incubated with blocking solution (3% H_2O_2 in PBS) for 5 minutes at room temperature.
7. The slides were rinsed twice with PBS, 5 minutes each time.
8. Tap off excess liquid and blot around the section
9. 75 μl of EQUILIBRATION BUFFER was applied on each section, the slides were incubated at room temperature for 5 minutes (the company recommend incubate at least 10 seconds).
10. Tap off excess liquid and blot around the sections
11. Each slide was covered with 50 μl WORKING STRENGTH OF TdT ENZYME (35 μl of reaction buffer + 15 μl TdT enzyme, 50 μl per slide), the sections were covered with the plastic covers came with the kit. The slides were incubate at 37°C for 60 minutes

12. The slides were placed in a staining jar that contained WORKING STRENGTH STOP/WASH BUFFER (1 ml STOP/WASH BUFFER + 34 ml H2O), the jar was agitated for 15 seconds, then incubated for 10 minutes at room temperature.
13. The slides were washed in PBS for 3 times, 1 minute each time.
14. Each section was covered with 65 µl of ANTI-DIGOXIGENIN PEROXIDASE CONJUGATE, slides were covered and incubated at 37°C for 30 minutes.
15. The slides were washed in 4 changes of PBS, 2 minutes per change.
16. Each section was covered with 75 µl of DAB solution, slides were incubated at RT for 3 to 6 minutes.
17. The slides were rinsed in 3 changes of tap water, and then incubated in running water for 5 minutes.
18. The slides were counterstained in Methyl Green (Vector, H3402) in a staining jar at 60 °C for 20 minutes.
19. The slides were washed in running tap water for 5 minutes.
20. The slides were dipped 5 times in 100% n-butanol, then dipped in 3 changes of Hemo-de, 10 dips each change (the exact times of dip have to be optimized to get a countable background).
21. The slides were mounted.
22. All slides were examined under a light microscopy. At least 2,000 hepatocytes were counted each slide, and the incidence of cell undergoing apoptosis was expressed as the percentage of number of stained hepatocytes out of the number of total counted hepatocytes.

Western blotting:

Reagents:

1. 1 M Tris-HCl, pH 6.8
2. 1 M Tris-HCl, pH 7.4
3. 1.5 M Tris-HCl, pH 8.8
4. 5 M NaCl
5. 15% Sodium deoxycholate
6. 1 M NaF

7. 1 M Na_2VO_3
8. 10% Sodium dodecyl sulfate (SDS) (w/v)

   10 g SDS (Sigma Chemical Co. L4390)

   100 ml diH_2O
9. 0.1 M DTT
10. 10 mg/ml Phenylmethylsulfonyl fluoride (PMSF), (Boehringer Mannheim 837 091)

    Dissolved in 100% ethanol
11. 0.33 µg/µl protease inhibitor cocktail

    1 mg aprotinin (Sigma Chemical Co. A1153)

    1 mg leupeptin (Sigma Chemical Co. L2884)

    1 mg pepstatin A (Sigma Chemical Co. P4265)

    Dissolved in 3 ml of autoclaved water and aliquoted. (pepstatin A was dissolved in 100 µl DMSO first before adding to cocktail)

Buffers:

1. 1 x Phosphate Buffered Saline (PBS), pH 7.4

Reagent	For 100 ml	Final concentration
Dibasic sodium phosphate (Na_2HPO_4) (Sigma Chemical Co. S5136)	0.1292 g	9.1 mM
Monobasic sodium phosphate (NaH_2PO_4) (Sigma Chemical Co. S5011)	0.0204 g	1.7 mM
Sodium Chloride (NaCl) (Sigma Chemical Co. S3014)	0.8766 g	150 mM

Adjusted pH to 7.4 with NaOH and brought final volume to 100 ml

2. Lysis/homogenization buffer for IKKs and IκBs

Reagent	For 25 ml	Final Concentration
Nonidet P-40 (Sigma Chemical Co. N6507)	0.25 ml	1%
10% SDS	0.25 ml	0.1%
10 mg/ml PMSF	0.25 ml (add last)	0.1 mg/ml
Protease inhibitors cocktail	133 μl (add last)	2 μg/ml
1x PBS	24.12 ml	

3. Lysis buffer for CYP2B1/2, cyclin D1 and Bcl-x_L

Reagent	For 20 ml	Final Concentration
1 M Tris-HCl, pH 7.4	1 ml	50 mM
5 M NaCl	0.6 ml	150 mM
IGEPAL	0.2 ml	1%
10% SDS	0.2 ml	0.1%
15% Sodium deoxycholate	0.667 ml	0.5%
1 M NaF	1 ml	50 mM
1 M Na_2VO_3	0.02 ml	1 mM
0.1 M DTT	0.2 ml	1 mM
0.1 M PMSF	0.2 ml	1 mM
0.33 mg/ml Protease inhibitors cocktail	0.3 ml (add last)	5 μg/ml
H_2O	15.613 ml	

4. Gel loading buffer (2x): according to Santa Cruz

Reagent	For 5.75 ml	Final Concentration
Glycerol (Sigma Chemical Co. G5516)	1.0 ml	17.3%
14.3 M β-mercaptoethanol (Sigma Chemical Co. M6250)	0.5 ml	1.25 M
10% SDS	3 ml	5.2%
1 M Tris pH 6.8	1.25 ml	0.22 M
1-2 mg bromophenol blue		

A stock can be made and stored at -20°C with the β-mercaptoethanol.

5. 10x Running buffer:

Reagent	For 250 ml	Final Concentration
Tris base	7.575 g	250 mM
Glycine (Gibco 15527-013)	36 g	1.92 M
SDS	2.5 g	1%

6. 1x Transfer Buffer:

Reagent	For 1 liter	Final concentration
Glycine	14.15 g	188 mM
Tris base	3.0 g	24.8 mM
100% methanol	200 ml	20%

Made fresh and kept at -20°C until use

7. 10x TBS (pH 7.5):

Reagent	For 1 liter	Final concentration
Tris-base	12.1 g	10 mM
NaCl	87.6 g	150 mM

pH with Hal to 7.5

8. Wash buffer (TTBS):

   1x TBS (1 liter)

   500 µl Tween-20 (0.05% final concentration) (Sigma Chemical Co. P7949)

9. 5% Blocking buffer (w/v):

   10 g fat-free dry instant powdered milk

   200 ml TTBS buffer

10. 10x Ponceau S stain

    2 g Ponceau S (Sigma Chemical Co. P3504)

    30 g trichloroacetic acid (Sigma Chemical Co. T4885)

    30 g sulfosalicyclic acid (Sigma Chemical Co. S0640)

    100 ml H_20

Procedure:

1. Sample preparation

   The livers were homogenized in 4 folds volume of lysis buffer for 3 x 30 second intervals with a 20 second rest in ice between the intervals. The homogenates for the IKKs and IκBs were centrifuged at 100,000 g for 1 hour; the homogenates for the CYP2B1/2, Bcl-x_L and cylcin D1 were centrifuged at 12,000 rpm for 15 minutes. The supernants were collected and the protein concentrations were checked using the BCA method (Pierce). Samples were aliquoted and frozen at -80°C. Aliquots were mixed with equal volume of the gel-loading buffer (2 x) and placed in boiling water for 3-5 minutes to denature the proteins before loading.

2. Gel electrophoresis/transfer of proteins

   An 8.5% separating gel and 4% stacking gel were cast in the frame. 25 μg of protein was electrophoresed per sample. A molecular weight marker (10 μl) was run with the samples (Bio-Rad 161-0324). The marker was boiled for 3-5 minutes before using. The proteins were electrophoresed with 1x running buffer at 175 volts for 2 hours. The proteins in the gels were transferred to nitrocellulose membranes (Gibco 11467-016) at 100 volts for 1 hour.

3. Immunoblotting

   The membranes were blocked with 5% blocking buffer for 1 hour at room temperature with shaking. The membranes were rinsed for five minutes with wash buffer before incubating with the primary antibody. The primary antibody was diluted in 5% blocking buffer (20 ml total) and incubated with the membrane for 1 hour with shaking at room temperature. The membrane was rinsed with wash buffer 3 times (5, 5, and 15 minutes). The secondary antibody was diluted in 5% blocking buffer (20 ml total) and incubated with the membrane for 1 hour with shaking at room temperature. The membrane was rinsed 3 times (5, 5, and 15 minutes) with the wash buffer. The following primary and secondary antibodies were used in this study:

Primary antibodies	Secondary antibodies
IκBα Santa Cruz sc-371 (1:1000 dilutions)	Anti-rabbit HRP antibody Santa Cruz sc-2004 (1:5000 dilution)

IκBβ Santa Cruz sc-945 (1:1000 dilutions)	Anti-rabbit HRP antibody Santa Cruz sc-2004 (1:5000 dilution)
IKKα/β Santa Cruz sc-7607 (1:1000 dilutions)	Anti-rabbit HRP antibody Santa Cruz sc-2004 (1:5000 dilution)
IKKγ Santa Cruz sc-8330 (1:1000 dilutions)	Anti-rabbit HRP antibody Santa Cruz sc-2004 (1:5000 dilution)
Cytochrome P450 2B1/2 Oxford Biomedical Research PM20 (1:1000 dilutions)	Anti-mouse HRP antibody Santa Cruz sc-2005 (1:5000 dilutions)
Cyclin D1 Santa Cruz sc-8396 (1:1000 dilutions)	Anti-mouse HRP antibody Santa Cruz sc-2005 (1:5000 dilutions)
Bcl-xL Santa Cruz sc-7195 (1:1000 dilutions)	Anti-rabbit HRP antibody Santa Cruz sc-2004 (1:5000 dilution)
β-Actin Santa Cruz sc-1615 (1:1000 dilutions)	Anti-goat HRP antibody Santa Cruz sc-2768 (1:5000 dilutions)

1. Chemiluminescence
    a. 10 ml of the peroxide solution and luminol/enhancer solution from Pierce SuperSignal West Pico Chemiluminescent Substrate Kit (34080) were mixed together and added to each membrane.
    b. Membrane was incubated with the chemiluminescent mixture for 5 minutes. The detection reagents were drained, and the membranes were covered in plastic wrap and exposed to Kodak XOMAT-AR film for 1 minute. The exposure time can be optimized based on original intensity.
    c. The membranes were placed back into the wash buffer and later stripped of the antibodies and detection reagents.
    d. The procedure for stripping the membranes was as follows: in a 50 ml tube, 42.55 ml of 0.235 M Glycine pH 2.2, 5 ml 5M NaCl, 2.45 ml sH_2O were mixed together. The membranes were placed in a pre-heated hybridization oven for 5 minutes at 60°C. The membranes were removed from the glycine buffer and

washed with 0.1M NaOH for 10 minutes at room temperature with shaking. The membranes were rinsed in wash buffer and the proteins were checked with 1x Ponceau stain. The membrane was either dried and stored or re-probed with another antibody.

RNA isolation:

Rageants :

1. Trizol Reagent – Life Technologies 15596
2. Chloroform
3. Ethanol – Aaper
4. Isopropyl alcohol – Sigma I-9516
5. Distilled water RNase, DNase-free – Gibco 10977-015

Procedure:

1. Homogenization

   60-80 mg liver tissue was homogenized 1 ml Trizol reagent for 30 seconds using a Tekmar homogenizer at 70 output. Incubated homogenized sample at room temperature for 5 minutes. If needed, stored samples at -80°C.

2. Phase separation

   The homogenized samples were transferred to a microcentrifuge tube, and 200 µl of chloroform was added. The mixture was shaken vigorously for 15 seconds and incubated at RT for 3 minutes. The samples were centrifuged at 12,000 x g for 15 minutes at 4°C.

3. RNA precipitation

   The top aqueous phase was transferred to a new microcentrifuge tube. 0.5 ml isopropyl alcohol was added and mixed gently. The tubes were incubated at RT for 10 minutes. The tubes were centrifuged at 12,000 x g for 10 minutes at 4°C.

4. RNA wash

   The supernatant was discarded, and tubes were allowed to drain on a clean Kim-wipe. 1 ml 75% ethanol was added. The tubes were vortexed and centrifuged at 7,500 x g for 5 minutes at 4°C.

5. Redissolving the RNA

The 75% ethanol was removed and the pellet was air-dried 10 minutes. Caution was taken to avoid over drying the pellet. The pellets were dissolved in 100 µl of DNase/RNase-free water. The RNA was incubated for 10 minutes at 55°C to dissolve completely. The dissolved RNA was aliquoted, and one aliquot was used to determine the RNA concentration and purity. The other aliquots were placed -80°C freezer until needed.

6. Quantitation and purity of RNA

   The purity of RNA was estimated by reading diluted RNA (1 part of RNA:200 parts of TE) with a spectrophotometer.

   The A_{260}/A_{280} for RNA was around 2.

   Total RNA = $(A_{260})(40\ \mu g/ml)(200)$

<u>Ribonuclease protection assay</u>:

Reagents *(all molecular biology grade):*

1. [α-^{32}P]UTP (3000Ci/mmol, 10 mCi/ml) – NEN BLU007H
2. Tris-saturated phenol, pH 8.0 (Sigma Chemical Co. P4557)
3. Chloroform:isoamyl alcohol (50:1)

   10 ml chloroform (Sigma Chemical Co. C2432)

   200 µl isoamyl alcohol (ICN 194005)
4. Mineral oil (Sigma Chemical M5904)
5. 10X TBE (Gibco 15581-044)
6. 40% Acrylamide (w/v) (Gibco 15512-023)
7. 2% N,N"-Methylenebisacrylamide (w/v) (Gibco 15516-016)
8. Urea (Gibco 15505-035)
9. Ultrapure, RNase-free H_2O (Gibco 10977-015)
10. 100% Ethanol (Aaper)
11. Ammonium persulfate (Sigma Chemical Co. A9164)
12. Dimethyldichlorosilane (Sigma Chemical Co. D3879) keep in 4°C
13. TEMED (Sigma Chemical Co. T7024)

Supplies:

1. RiboQuant ™ RNase Protection Assay Kit - Becton Dickinson Pharmingen

556134

2. MCYC-1 RiboQuant ™ Mouse Cyclin Multi-Probe Template set – Becton Dickinson Pharmingen 556241
3. RiboQuant ™ In Vitro Transcription Kit – Becton Dickinson Pharmingen 556850
4. RNase-free 1.7 ml microcentrifuge tubes, pipette tips, and plastic ware
5. RNase-free glassware (baked for at least 4 hours at 150°C)
6. Gel blotting paper – Schleicher & Schuell # 31540

Probe Synthesis:

1. The following were added to a microcentrifuge tube:

   1 µl RNasin
   1 µl GACU pool
   2 µl DTT
   4 µl 5x transcription buffer
   1 µl RPA Template set (Pharmingen mCYC-1 RiboQuant Multi-Probe 556241)
   10 µl [α-^{32}P]UTP
   1µl T_7 RNA Polymerase

   The contents were mixed by flicking and given a quick spin in a microcentrifuge. The reaction was incubated for one 1 hour at 37°C in heat block.

2. To stop the reaction, 2 µl of DNase were added to the reaction. The tube was mixed by flicking and spun quickly. The tube was incubated 30 minutes at 37°C.
3. The following were added to the microcentrifuge tube:

   26 µl 20 mM EDTA

   25 µl Tris-saturated phenol, pH=8.0

   25 µl chloroform:isoamyl alcohol (50:1)

   2.6 µl tRNA

   The tube was mixed by vortexing into an emulsion and spun at 12,000 rpm in microcentrifuge for 5 minutes at room temperature.

4. The upper aqueous phase was transferred to a new microcentrifuge tube. 50 µl chloroform:isoamyl alcohol was added. The tube was mixed by vortexing and spun at room temperature for 2 minutes at 12,000 rpm.
5. The upper aqueous phase was transferred to a new tube. In order to precipitate the RNA probe, 50 µl 4 M ammonium acetate and 250 µl ice-cold 100% ethanol were

added to the tube. The tube was inverted and incubated overnight at -80°C. The tube was spun at maximum speed in a microcentrifuge for 15 minutes at 4°C.

6. The supernatant was removed and added 100 µl of cold 90% ethanol was added to the small pellet. The tube was spun in a microcentrifuge for 5 minutes at 4°C.
7. Being careful not to disturb the pellet, the supernatant was removed and the pellet was air dried for 10 minutes. The pellet was dissolved in 50 µl of the hybridization buffer and vortexed gently for 20 seconds followed with a quick spin.
8. Using a scintillation counter, 1 µl of the probe was quantified. The average counts of the labeled probe were $2x10^5$ Cherenkov counts/µl (measurement of cpm/µl in the absence of scintillation fluid). The probe was stored at -20°C and was usable up to a week.

RNA Preparation and Hybridization:

9. One aliquot of each sample of RNA was removed from -80°C freezer and 15 µg of RNA were added to a new microcentrifuge tube. As a background control, one tube containing 1.2 µl of yeast tRNA. To serve as a positive RNA integrity control, another tube contained 2 µl of the PharMingen mouse control RNA (556213).
10. The RNA was placed in -80°C freezer for 15 minutes. The RNA was dried in a vacuum evaporator centrifuge for 30 minutes without heat.
11. 8 µl of hybridization buffer was added to each sample and vortexed for 3 minutes followed with a quick spin.
12. The mCYC probe was diluted to 1.15 x 10^5 count/µl. 2 µl of diluted probe was added to each RNA sample and mixed by gently flicking the tube. One drop of mineral oil was added to each tube and all tubes were given a quick spin.
13. All samples were placed in a 90°C heat block. The temperature was immediately adjusted to 56°C, and the samples were incubated 12-16 hours. Fifteen minutes before starting the RNase treatments, the heat block was adjusted to 37°C, allowing the temperature to ramp down slowly.

RNase Treatments:

14. For 26 samples, prepared the RNase cocktail:

2.8 ml RNase buffer + 6.0 µl (RNase + T1 mix) were combined and 100 µl of the cocktail was added to each sample (under the mineral oil). All samples were given a quick spin and incubated at 30°C for 45 minutes.

15. During the RNase digestion, the Proteinase K cocktail was prepared. For 26 samples the following were combined:

    421.2 µl Proteinase K buffer

    32.4 µl Proteinase K

    32.4 µl yeast tRNA

    All mixed together and 18 µl cocktail was added to a new tube for all samples

16. 105 µl of the RNase digests were carefully removed and transferred to the 18 µl aliquot of Proteinase K cocktail. The samples were quickly vortexed spun, and incubated for 15 minutes at 37°C.

17. The following were added to each sample:

    65 µl Tris-saturated phenol

    65 µl chloroform:isoamyl alcohol (50:1)

    The mix was vortexed and spun in microcentrifuge for 5 minutes at room temperature at max speed

18. The upper aqueous phase (120 µl) was transferred to a new tube (avoiding organic interface), and 120 µl 4 M ammonium acetate and 650 µl ice-cold 100% ethanol were added. The tubes were inverted and incubated for 30 minutes at -70°C. The tubes were spun at maximum speed for 15 minutes at 4°C.

19. Avoiding the pellet, the supernatant was removed. 100 µl ice-cold 90% ethanol was added the supernatant, and the tubes were spun at maximum speed for 15 minutes at 4°C in microcentrifuge.

20. The supernatant was carefully removed and the pellets were air-dried. 5 µl of the 1x loading buffer was added to each tube, vortexed for 2 minutes, and given a quick spin in a microcentrifuge. Before loading samples for electrophoresis, the samples were heated at 90°C for 3 minutes and placed into an ice bath.

Electrophoresis:

21. A set of clean glass plates (19 cm x 19.7 cm) was rinsed thoroughly with RNase free water then with ethanol. The short plate was siliconized and cleaned again. The plates were assembled.
22. A 5% acrylamide gel (19:1 acrylamide/bis) was prepared (45 ml):

    5.31 ml 40% acrylamide

    5.58 ml 2% bis acrylamide

    4.47 ml 10X TBE

    21.5 g Urea (8M final)

    brought volume to 44.69 ml with DEPC-treated water

    270 µl 10% ammonium persulfate (APS)

    36 µl TEMED

    The acrylamide mixture was poured immediately into gel assembly. The gel polymerized for at least 1 hour.
23. The comb was removed and the wells were thoroughly flushed with 0.5X TBE. The gel was pre-run for 45 minutes at 250 volts with 0.5X TBE as the running buffer. To avoid overheat, the gel was run at 4°C.
24. Before loading the samples, the wells were flushed again with 0.5X TBE. The samples were loaded into each well, and a dilution of free probe was run with the samples (in 1x loading buffer) to serve as a size marker (1000-2000 cpm/lane).
25. The gel was run at 300 volts for 2 ½ hours. The first dye band (bromophenol blue) ran off the gel.
26. The gel plates were disassembled, and the gel was transferred to pre-cut gel blotting paper. The gel was wrapped with plastic wrap dried on a gel dryer (Owl Scientific #GD-3) under vacuum for 1 hour at 80°C. The dried gel was placed on a phosphorimaging screen (Molecular Dynamics) for at least 4 hours. The screen was scanned into a phosphorimager, and the radioactivity was counted using the ImageQuant 5.0 software.
27. Using semi-log graph paper, the undigested (free) probe was plotted with the migration on the x-axis and the log nucleotide length on the y-axis, thus creating a standard curve. The migration of the RNase-protected bands was plotted on the

standard, and the nucleotide length was calculated (estimated). This value was compared to the actual length of each protected probe, thereby correctly identifying each band.

Statistical analysis:

Results were first analyzed by a two-way analysis of variance. Individual differences between means were determined using the Bonferroni post hoc test. The results were reported as means $\pm$ standard error of mean (SEM). The level of significance was $p \leq 0.05$.

Results

Body weight and liver weight

In the 2-day study, there was no significant difference in the body weight or liver weight among all groups (Table 3.1). In the 21-day study, the body weights of p50-/- mice were significantly less than those of wild type mice. PCB-153 treatment significantly increased the liver weight and relative liver weight (percentage of body weight) in both p50-/- mice and wild type mice.

Hepatic cytochrome P450 2B1/2 (CYP2B1/2) protein

The protein level of CYP2B1/2 in the liver was measured with Western blotting. There was no difference in the hepatic protein level of CYP2B1/1 between p50-/- mice and wild type mice (Figure 3.1). PCB-153 slightly increased the protein level of CYP2B1/2 in wild type mice and in p50-/- mice in both studies.

Hepatic NF-κB activity

In the 2-day study, the hepatic DNA binding activity of NF-κB was significantly increased by PCB-153 in wild type mice (Figure 3.2), but not in p50-/- mice. The protein levels of IKKs (IKKα, IKKβ, IKKγ) and IκBβ were not changed by PCB treatment, and there was no difference in these proteins between wild type mouse and p50-/- mouse (Figure 3.3). There was less IκBα protein in p50-/- mouse than in wild type, but IκBα protein was not affected by PCB-153 treatment.

In the 21-day study, the hepatic DNA binding activity of NF-κB was higher in wild type mice than p50-/- mice (Figure 3.4), and there was no difference in NF-κB activity between PCB-treated animals and corn oil-treated controls. As in the 2-day study, the protein levels of IKKs (IKKα, IKKβ, IKKγ) and IκBβ were not changed by PCB treatment (Figure 3.5), and there was no difference in these proteins between wild type and p50-/- mice. There was less IκBα protein in p50-/- mice than in the wild types, and IκBα protein was not affected by PCB-153 treatment.

Hepatocyte proliferation and apoptosis

In the 2-day study, PCB-153 treatment increased hepatocyte proliferation in wild type mice significantly ($p < 0.05$), but not in p50-/- mice (Figure 3.6). In the 21-day study, PCB-153 treatment increased hepatocyte proliferation significantly in wildtypes ($p < 0.05$); in the p50-/- mice there was a slight increase (Figure 3.7).

Apoptosis in hepatocytes was measured by the TUNEL assay. The apoptotic index was much higher in p50-/- mice than in wild type mice ($p < 0.05$), and this increase was blocked by PCB-153 in both studies (Figure3.8, 3.9), with a more pronounced effect in the 21-day study ($p < 0.05$).

mRNA and protein levels of cyclins

The mRNA levels of cyclin A2, B1, B2, C, D1 and D2 in liver were measured using RNase protection assay (RPA) (Table 3.2). For all the cylcins studied, there were no significant differences between wild type mice and p50-/- mice in both studies. PCB-153 significantly decreased the mRNA level of cyclin A2, B1, B2, and C in the 2-day study ($p < 0.05$), but not that of cyclin D1 or D2; in the 21-day study, the mRNA level of all cyclins were not significantly different among the groups.

The protein level of cyclin D1 was measured with Western blotting. Compared to wild type mice, p50-/- mice had less cyclin D1 protein in the liver (Figure 3.10). The protein level of cyclin D1 in the liver was not changed by PCB-153 in either study.

Bcl-xL protein

The protein level of Bcl-xL in the liver was measured with Western blotting. In either study, there was no difference in the protein level of Bcl-xL in the liver among any groups (Figure 3.11).

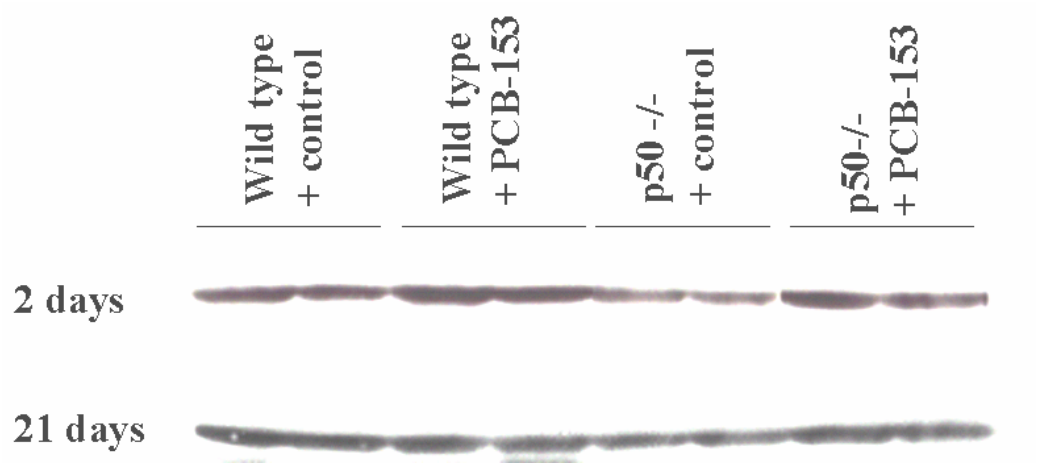
Table 3.1 Effect of PCB-153 on the body and liver weights of the p50-/- mice and wild type mice

Genotype	Treatment	Body weight (g)	Liver weight (g)	Liver weight (% of body weight)
2 days				
Wild type	corn oil	25.64 ± 0.73	1.32 ± 0.07	5.15 ± 0.27
	PCB-153	25.51 ± 0.50	1.48 ± 0.06	5.79 ± 0.15
p50-/-	corn oil	24.27 ± 0.99	1.53 ± 0.09	6.30 ± 0.18
	PCB-153	22.61 ± 0.61	1.39 ± 0.05	6.12 ± 0.08
21 days				
Wild-type	corn oil	29.65 ± 0.62	1.55 ± 0.06	5.24 ± 0.18
	PCB-153	31.41 ± 0.91	1.77 ± 0.10^{+}	5.63 ± 0.25^{+}
p50-/-	corn oil	25.65 ± 1.24*	1.54 ± 0.09	5.99 ± 0.11*
	PCB-153	25.37 ± 1.21*	1.71 ± 0.09^{+}	6.73 ± 0.20^{+}*

Results are expressed as mean ± SEM. Each group contained 5-10 animals. *Values are significantly different from wild type mice. $^{+}$Values are significantly different from respective controls treated with corn oil. ($p < 0.05$).

Figure 3.1. Protein levels of CYP2B1/2 in the 2-day and 21-day studies. A. Western blot was performed using an antibody specific for CYP 2B1/2 with liver homogenates from mice treated with corn oil, or PCB-153 (300 μmol/kg in the 2-day study, 100 μmol/kg for 6 injections in the 21-day study). Each lane contains protein from a single mouse (25 μg). B. Relative amount of CYP 2B1/2 protein from the Western blotting data as shown in A.

A.


Wild type + control
Wild type + PCB-153
p50 -/- + control
p50 -/- + PCB-153
2 days
21 days

B.

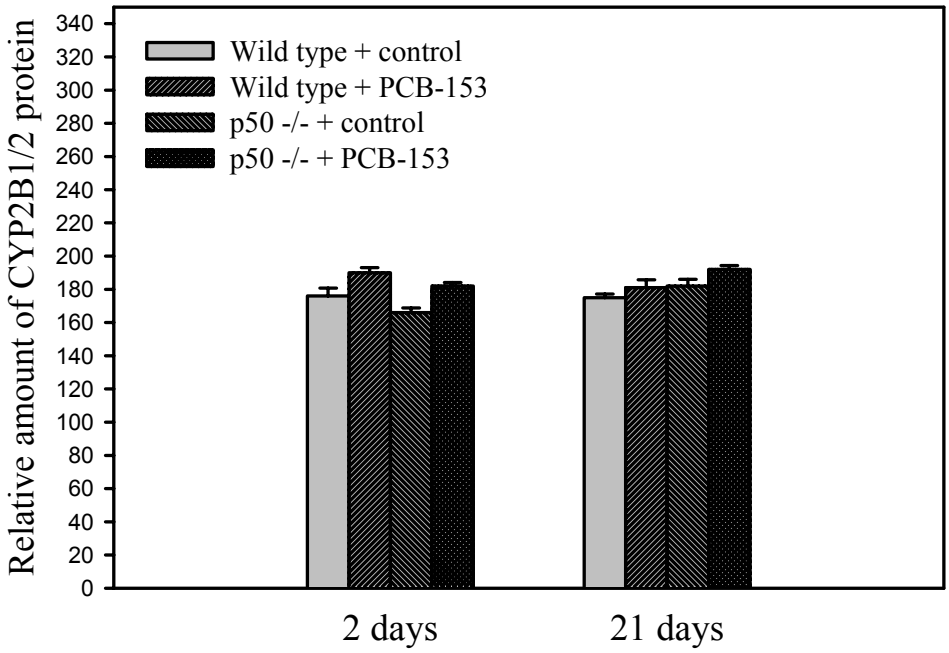
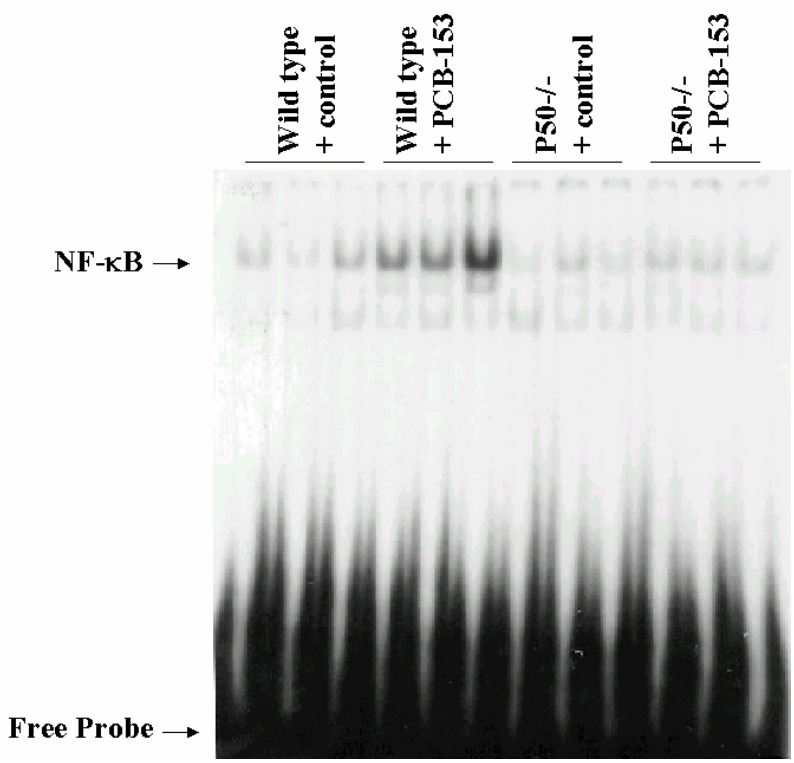

Relative amount of CYP2B1/2 protein
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
Wild type + control
Wild type + PCB-153
p50 -/- + control
p50 -/- + PCB-153
2 days
21 days

Figure 3.2. Effect of PCB-153 on hepatic DNA binding activity of NF-κB in the 2-day study. A. EMSAs were performed using a radiolabeled NF-κB oligonucleotide with liver nuclear extracts from individual mice. Each line contains extracts from a single mouse (5 μg). B. Net radioactive counts of NF-κB bands from the EMSA data shown in A. Quantitation of the specific NF-κB band was determined by subtracting background counts from the counts in each NF-κB band. *Values are significantly different from other groups ($p < 0.05$).

A


Wild type + control
Wild type + PCB-153
P50-/- + control
P50-/- + PCB-153
NF-κB
Free Probe

B

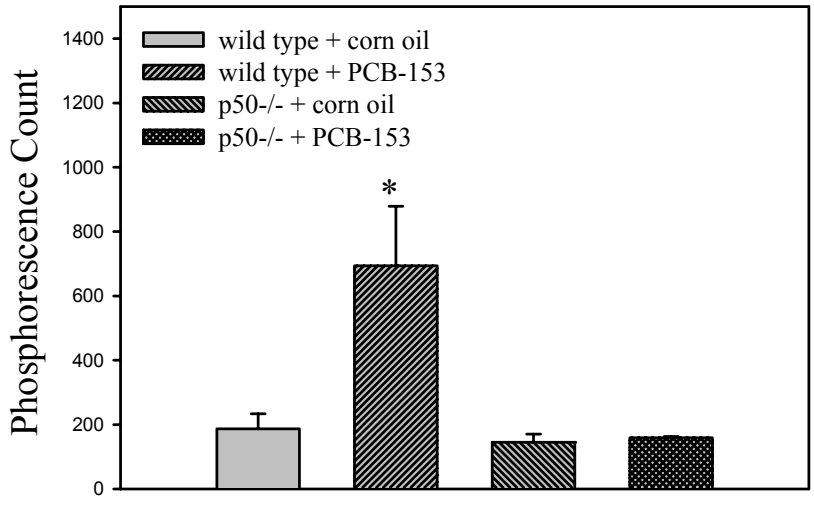

wild type + corn oil
wild type + PCB-153
p50-/- + corn oil
p50-/- + PCB-153
Phosphorescence Count
1400
1200
1000
800
600
400
200
0
*

Figure 3.3. Protein levels of cytosolic IκB and IKK in the 2-day study. Each line contains cytosolic protein from a single animal (25 μg).

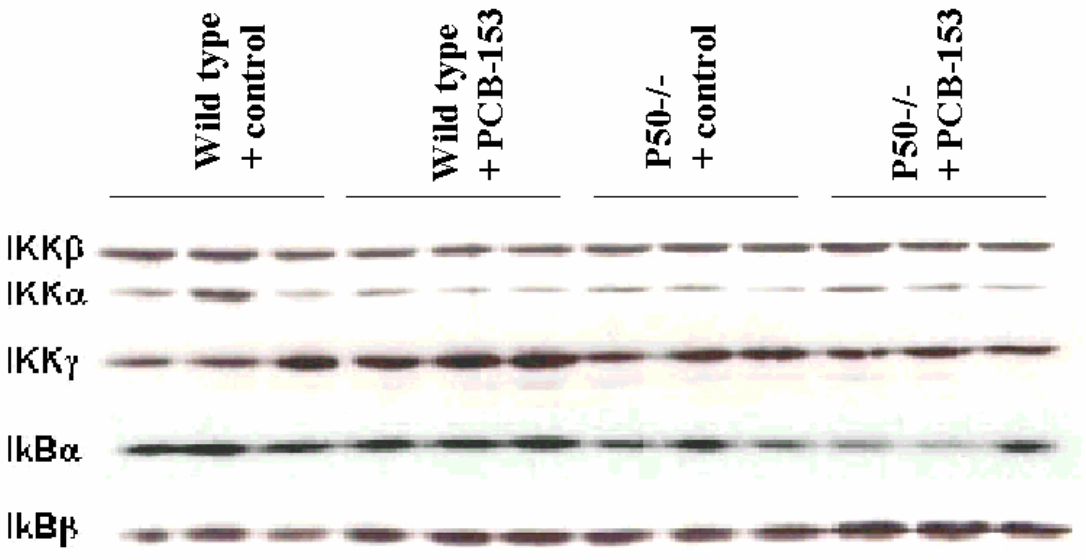
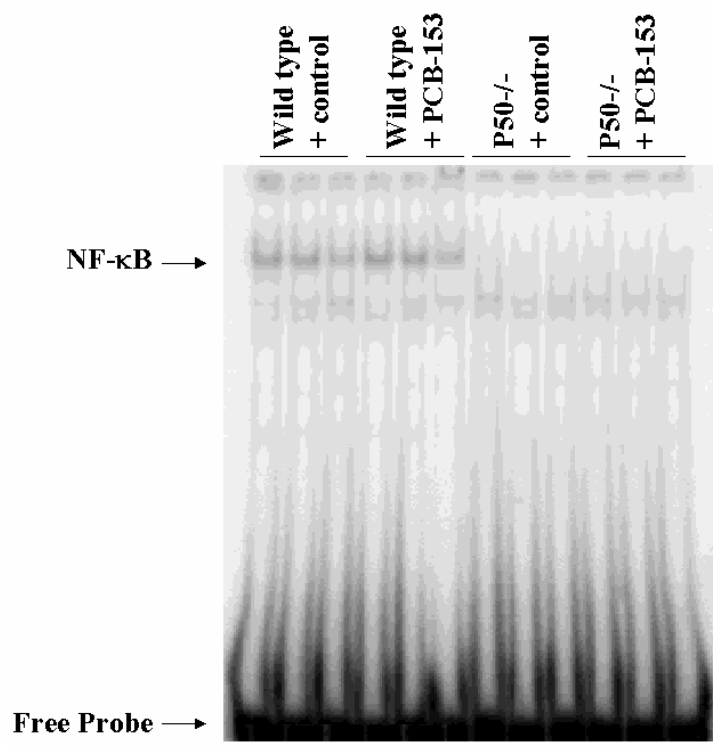


Figure 3.4. Effect of PCB-153 on the hepatic DNA binding activity of NF-κB in the 21-day study. A. EMSAs were performed using a radiolabeled NF-κB oligonucleotide with liver nuclear extracts from individual mice. Each line contained extracts from a single mouse (5 μg). B. Net radioactive counts of NF-κB bands from the EMSA data shown in A. Quantitation of the specific NF-κB band was determined by subtracting background counts from the count in each NF-κB band. *Values are significantly different from other groups ($p < 0.05$).

A


Wild type + control
Wild type + PCB-153
P50-/- + control
P50-/- + PCB-153
NF-κB
Free Probe

B

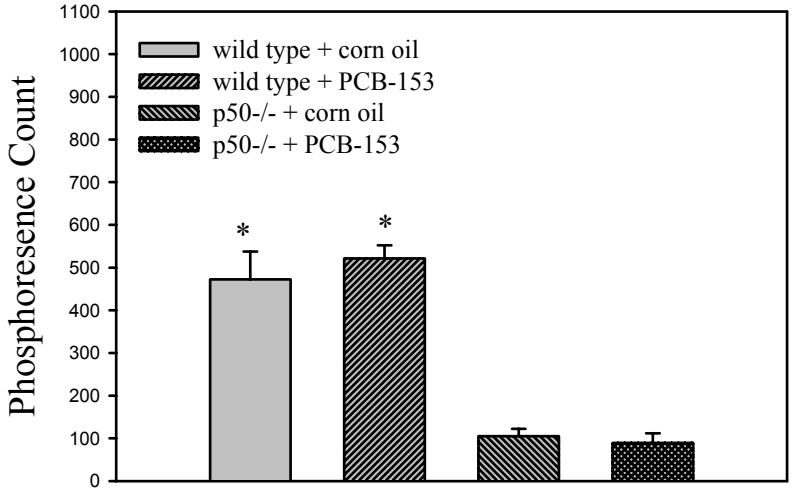

Phosphoresence Count
1100
1000
900
800
700
600
500
400
300
200
100
0
wild type + corn oil
wild type + PCB-153
p50-/- + corn oil
p50-/- + PCB-153
*
*

Figure 3.5. Protein levels of IκB and IKK proteins in the 21-day study. Each line contains cytosolic protein from a single mouse (25 μg).

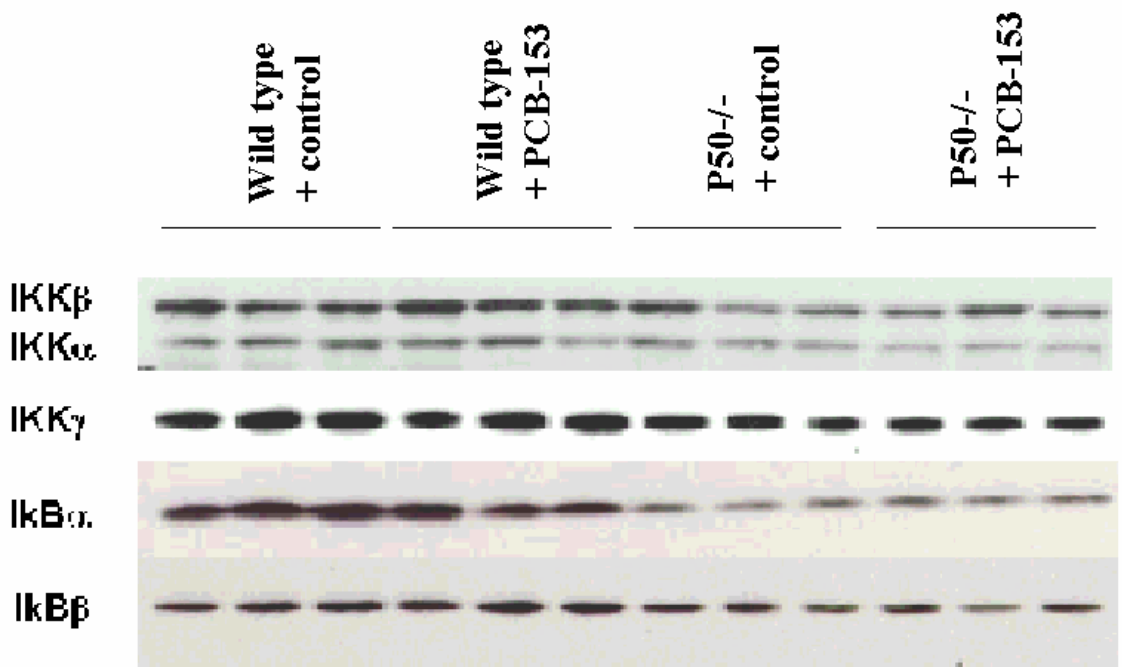


Figure 3.6. Effect of PCB-153 on hepatocyte proliferation in the 2-day study. Mice were administrated BrdU by a s.c. injection of BrdU two hours before the euthanasia. Tissue sections were immunohistochemically stained for BrdU and the labeling index was determined. Each group contained 5-10 animals. *Values are significantly different from other groups ($p < 0.05$).

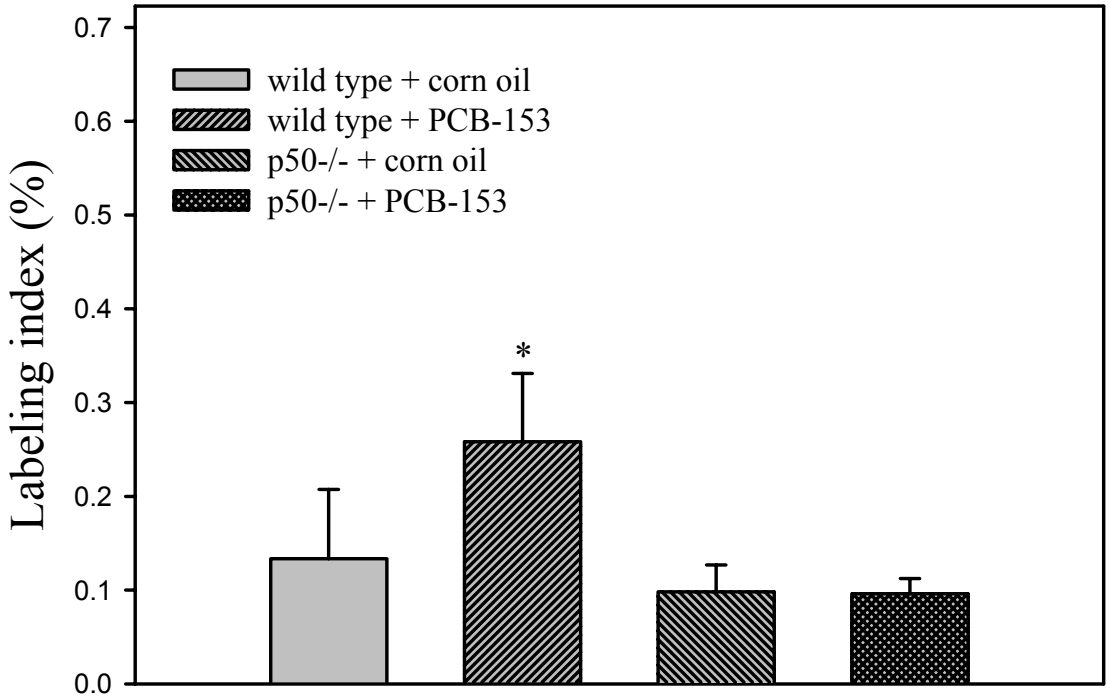

Labeling index (%)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
wild type + corn oil
wild type + PCB-153
p50-/- + corn oil
p50-/- + PCB-153
*

Figure 3.7. Effect of PCB-153 on hepatocyte proliferation in the 21-day study. Mice were administrated BrdU by a 3-day infusion using Alzet osmotic pump. Tissue sections were immunohistochemically stained for BrdU and labeling index was determined. Each group contained 5-9 animals. a, b Groups with different letters are significantly different ($p < 0.05$).

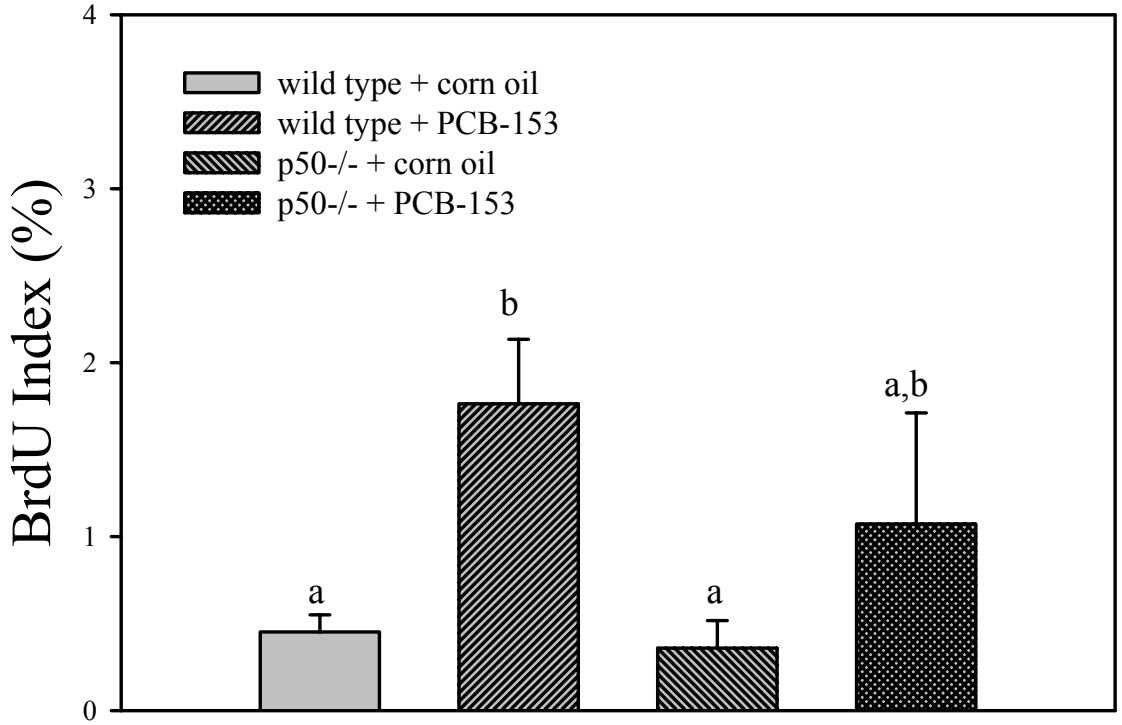

BrdU Index (%)
0
1
2
3
4
wild type + corn oil
wild type + PCB-153
p50-/- + corn oil
p50-/- + PCB-153
a
b
a
a,b

Figure 3.8. Effect of PCB-153 on hepatocyte apoptosis in the 2-day study. Liver section were immunostained using the TUNEL method, and apoptotic index was determined for each mouse. Each group contained 5-10 animals. *Values are significantly different from other groups ($p < 0.05$).

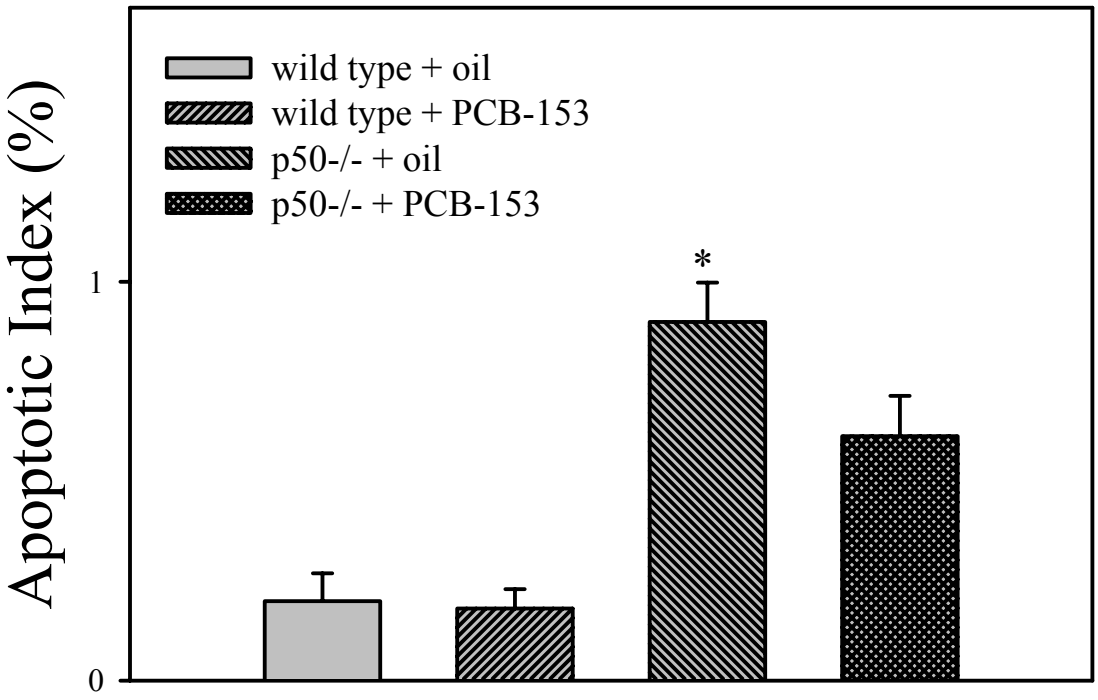

wild type + oil
wild type + PCB-153
p50-/- + oil
p50-/- + PCB-153
Apoptotic Index (%)
1
0
*

Figure 3.9. Effect of PCB-153 on hepatocyte apoptosis in the 21-day study. Liver section were immunostained using the TUNEL method, and apoptotic index was determined for each mouse. Each group contained 5-9 animals. a, b, c Groups with different letters are significantly different ($p < 0.05$).

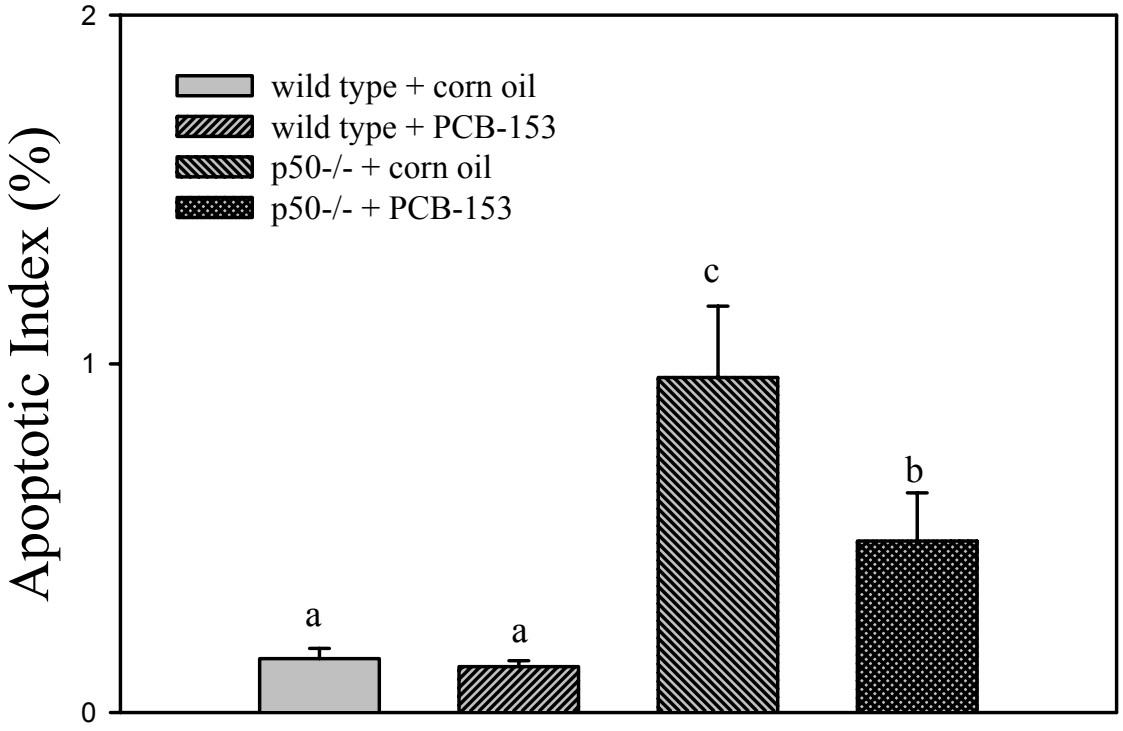

Apoptotic Index (%)
2
1
0
wild type + corn oil
wild type + PCB-153
p50-/- + corn oil
p50-/- + PCB-153
a
a
c
b

Table 3.2. Effect of PCB-153 on hepatic cyclin mRNA expression in p50-/- mice and wild type mice in the 2-day and 21-day studies

		cyclins mRNA (%)					
Genotype	Treatment	A2	B1	B2	C	D1	D2
2 days							
Wildtype	corn oil	15.86 ± 0.61	12.00 ± 0.52	29.45 ± 0.77	22.06 ± 0.65	21.01 ± 2.80	21.49 ± 0.34
	PCB-153	14.36 ± 0.45*	11.04 ± 0.75*	27.39 ± 0.95*	20.54 ± 0.22*	28.26 ± 3.78	19.95 ± 0.80
p50-/-	corn oil	16.65 ± 1.17	13.62 ± 1.36	29.43 ± 1.00	21.91 ± 0.86	22.11 ± 4.80	22.49 ± 0.55
	PCB-153	13.33 ± 0.40*	10.03 ± 0.37*	26.95 ± 5.30*	19.29 ± 0.08*	28.60 ± 4.68	20.52 ± 1.64
21 days							
Wildtype	corn oil	11.84 ± 0.56	10.13 ± 0.44	28.24 ± 1.59	16.95 ± 0.78	27.65 ± 4.05	21.99 ± 1.10
	PCB-153	12.51 ± 0.84	10.92 ± 0.56	27.66 ± 0.22	18.06 ± 0.84	20.33 ± 0.89	21.46 ± 0.89
p50-/-	corn oil	11.92 ± 0.69	10.15 ± 0.46	30.99 ± 0.45	18.81 ± 0.67	25.26 ± 2.78	22.08 ± 1.25
	PCB-153	11.81 ± 0.79	10.28 ± 1.15	29.29 ± 2.96	18.36 ± 1.58	22.17 ± 2.50	21.76 ± 1.97

Results are expressed as the percentage of the control (L32). Numbers are shown as the mean ± SEM with n = 3 for each group. *Values are significantly different from respective corn oil-treated controls ($p < 0.05$)

Figure 3.10. Protein level of cyclin D1 in the 2-day and 21-day studies as determined by Western blotting. Each line contains protein from a single mouse (25 μg).

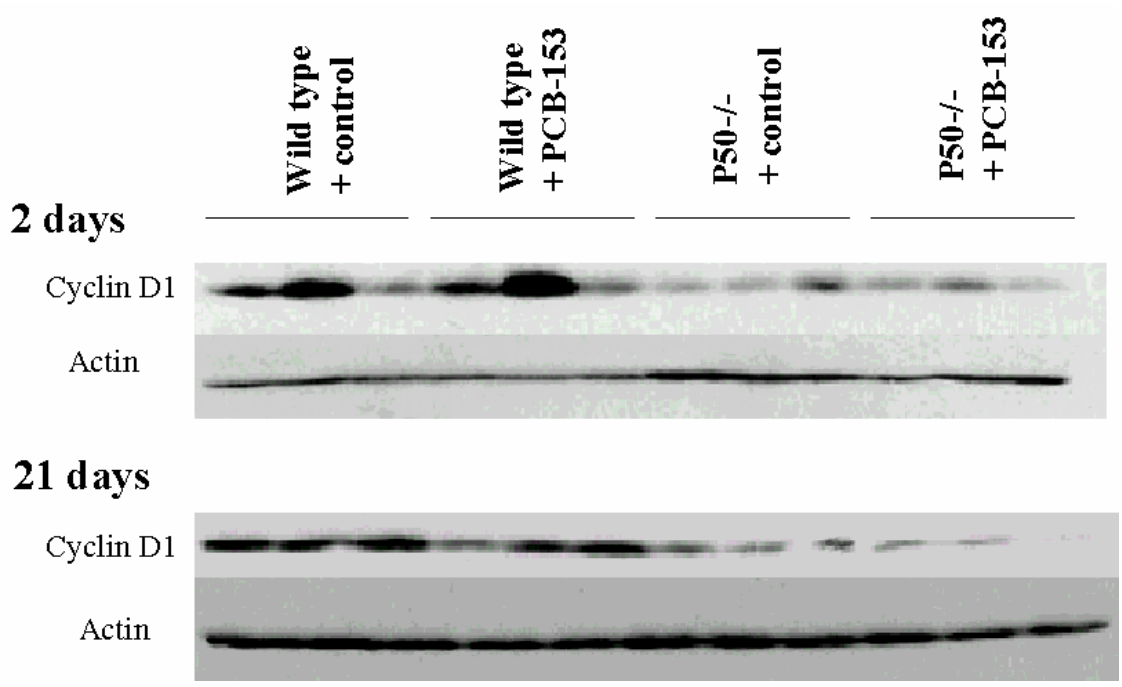
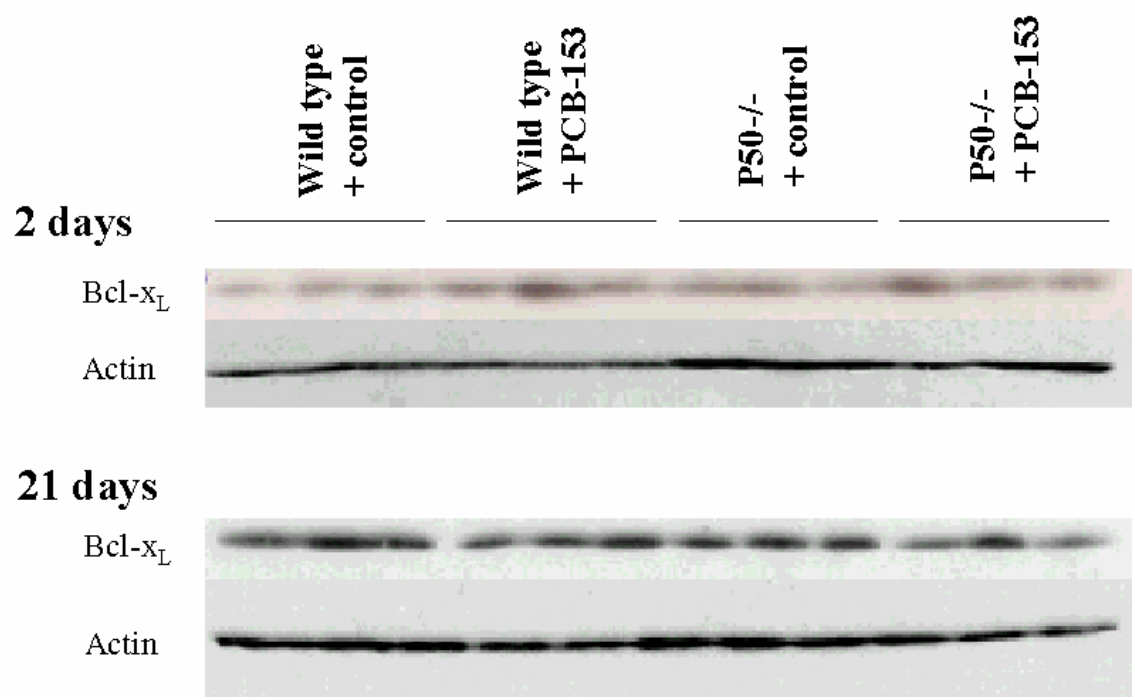


Figure 3.11. Protein level of Bcl-x_L in the 2-day and 21-day studies as determined by Western blotting. Each line contains protein from a single mouse (25 μg).



Discussion

Recent studies have indicated that PCB mixtures and specific PCB congeners can cause an increase in cell proliferation and an inhibition of apoptosis in rodent liver or hepatocytes (Bohnenberger *et al*. 2001; Haag-Gronlund M 2000; Kolaja *et al*. 2000; Tharappel *et al*. 2002; Whysner & Wang 2001). PCB-118, PCB-77 and Aroclor 1254, a PCB mixture, have all been found to induce hepatocyte proliferation during hepatocarcinogenesis. Also, our previous work showed an increase in hepatocyte proliferation after a single dose of PCB-153 (Chapter 2). The purpose of this study was to elucidate the role of NF-κB in the regulation of hepatocyte proliferation and inhibition of apoptosis.

In the 2-day study, PCB-153 increased hepatic NF-κB activity by PCB-153 in wild type mice, but not in p50-/- mice, which was not surprising since both p50 and p65 are present in the NF-κB protein-DNA binding complex. The IKKs and IκBs proteins were no changed by PCB-153, suggesting the presence of an IκB-independent NF-κB activation pathway. The protein level of IκBα was lower in p50-/- livers compared to p50+/+ livers, possibly reflecting a compensatory response to the loss of p50/p65 activity. Unlike the 2-day study, the hepatic NF-κB activity in wild type mice was not induced by PCB-153 in the 21-day study, but the higher activity of NF-κB in these mice compared to p50-/- mice suggested stresses caused by the multiple i.p. injections in those mice.

PCB-153 increased hepatocyte proliferation in wild type mice, but not in p50-/- mice in the 2-day study, which showed that NF-κB (p50/p65) is important in the regulation of PCB-153-induced cell proliferation. Studies have shown normal liver regeneration in p50-/- mice (DeAngelis *et al*. 2001), as the result of a compensatory effect increase in the p65 subunit of NF-κB. In our 2-day study, p50-/- livers contained the same low level of NF-κB activity as in wild type livers; this could be explained by overexpression of p65 with the formation of p65 homodimers, or by the binding of p65 to other NF-κB family members. An increase in p65 or other NF-κB family members and the decreased amount of IκBα in p50-/- livers would explain the increase in DNA synthesis at later time point, as shown in the 21-day study. IκBα is an important target

gene of p50/p65 that down-regulates NF-κB activity. The reduced down-regulation of p65 could compensate for the absence of p50. However, p65 cannot fully compensate for the p50 deficiency, as shown in the low level of DNA synthesis in the single dose PCB-treated p50-/- livers and the higher incidence of apoptosis in p50-/- livers.

Hepatocytes typically proliferate at a very low rate, yet increased proliferation occurs in response to physical, infectious or toxic injury (Kitamura *et al*. 1998)]. The cell cycle control system is regulated by the expression of cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CKIs). Control of mammalian cell proliferation by extracellular signals takes place in the mid- to late- G1 phase of cell cycle. Cyclin D, in association with CDK4 and CDK6, as well as cyclin E/CDK2, promotes the G1-to-S phase transition by phosphorylating retinoblastoma protein (pRB), hyperphosphorylations of pRB release the transcription factor E2F, which is required for the activation of S-phase-specific genes (Bartek J 1996; Beijersbergen RL 1996). Transcriptional activation of the cyclin D1 gene occurs through the Ras signaling pathway via MAPKs, ERK1 and ERK2 (Lavoie *et al*. 1996). In addition, accumulating data show that NF-κB binding to the cyclin D1 promoter is critical for cyclin D1 transcription initiation and hyperphosphorylation of pRB (Hinz *et al*. 1999) (Guttridge *et al*. 1999; Henry *et al*. 2000; Joyce *et al*. 1999). The overexpressions of Cyclin D1 has been observed in hepatocellular carcinoma (HCC) (Joo *et al*. 2001); studies with transgenic mice model show that targeted overexpression of cyclin D1 leads to the development of mammary carcinomas (Wang *et al*. 1994) and hepatocellular carcinoma (Deane *et al*. 2001). Hepatocyte cyclin D1 expression can be increased by the hepatic tumor promoters nafenopin (Chevalier & Roberts 1999) and phenobarbital (Kinoshita *et al*. 2002), which suggests that changes in the cyclins/CDK complexes and the CKIs could result in the loss of regulation of hepatocyte proliferation. In our studies, we compared the change in the mRNA level of cyclin A2, B1, B2, C, D1 and D2. There was no significant change in the cyclin D1 mRNA level between wildtype and p50-/- livers, or after PCB-153 treatments, but there was less cyclin D1 protein in p50-/- livers than in the wild type livers. The lack of paralleled changes between mRNA and protein levels of cyclin D1 has been described early in regenerating liver after partial hepatectomy (Albrecht *et al*. 1995) and in adult rat liver (Awad & Gruppuso 2000). Cyclin D1 mRNA

was highly induced in rat liver after partial hepatectomy, while the protein levels changed < 2 fold and did not parallel changes in the mRNA (Albrecht *et al*. 1995). In adult rat livers, the mRNA levels of cyclin D1 were present as a higher level than in fetal livers, while the protein was absent in adult livers, in which a posttranscriptional regulation was indicated (Awad & Gruppuso 2000). How the absence of p50 protein decreases Cyclin D1 at the protein level but not the mRNA level remains unclear, but our data do suggest an important role of Cyclin D1 posttranslational regulation in hepatocyte growth regulation.

The suppression of apoptosis is another important mechanism by which nongenotoxic hepatocarcinogens may induce cancer. The tumor promoters phenobarbital and non-coplanar PCBs have been shown to inhibit UV-induced apoptosis in primary hepatocytes (Bohnenberger *et al*. 2001). Tharappel *et al*. have shown inhibition of apoptosis by PCB-153 in focal hepatocytes in an initiation-promotion study (Tharappel *et al*. 2002). Salvi *et al*. have shown that PCBs inhibit the mitochondrial permeability transition and the consequent release of cytochrome C from mitochondria (Salvi & Toninello 2001). Considering the pivotal role of cytochrome C in caspase activation, inhibition of its release would indicate an interruption of apoptotic pathway. However, there are few quantitative data on apoptosis in mouse liver and little is known about its regulation in hepatocytes. In the studies by DeAngelis *et al*. (DeAngelis *et al*. 2001), analysis of apoptosis in p50-/- livers showed a small increase in overall apoptosis in the absence of p50, in which Fas antibody was used to activate the Fas receptor pathway and induce apoptosis, but apoptosis in untreated livers was not reported in that study. In our study, we showed that a very low number of hepatocytes were undergoing apoptosis in wild type mice, while p50-/- livers showed more apoptosis. Although this increase in spontaneous apoptosis is not as severe as that in the p65 knockout mouse, which causes massive hepatocyte apoptosis and embryonic death (Beg *et al*. 1995), it demonstrated the importance of NF-κB (p50/p65) as anti-apoptotic mediators.

Studies have demonstrated that Bcl-2 family members were involved in the inhibition of apoptosis by phenobarbital and peroxisome proliferators in primary hepatocytes and mouse liver (Christensen *et al*. 1998; Christensen *et al*. 1999; Sanders & Thorgeirsson 1999), which suggested a possible mechanism of NF-κB as anti-apoptotic

mediator in liver. Bcl-2 family members are mitochondrial membrane proteins; Bcl-2 and Bcl-x_L inhibit release of cytochrome C from mitochondria and act as anti-apoptotic proteins, while Bax and Bak are apoptosis-inducing proteins. The precise mechanism by which these proteins regulate cell death is still under investigation. However, the cellular equilibrium between inducing and inhibiting proteins is important in determining cell fate. Both the human and mouse Bcl-x_L promoters contain NF-κB binding sites (Lee *et al*. 1999; Tamatani *et al*. 1999), and this binding of NF-κB is important in Bcl-x_L expression and for the anti-apoptotic effects at least in immune CD40 cells (Lee *et al*. 1999). But we did not find any change in the protein level of Bcl-x_L by PCB-153 in the present study, and there was no difference in the Bcl-x_L protein between p50-/- and wild type livers. Christensen *et al*. have shown that acute treatment with phenobarbital resulted in an induction of Bcl-2 protein levels in mouse livers, but Bcl-x_L was not changed (Christensen *et al*. 1999). However, the same study showed increased Bcl-x_L expression in altered hepatic foci (AHF), adenomas and carcinomas after long-term treatment with phenobarbital.

In summary, our data support the hypothesis that NF-κB activation plays a central role in regulation of cell proliferation and inhibition of apoptosis in liver, and that the activation of NF-κB by PCBs may contribute to the tumor promoting activity of non-coplanar PCBs congener PCB-153.

Chapter 4. Effect of dietary vitamin E on cell proliferation and transcription factor NF-κB activation during the promotion of liver carcinogenesis by Polychlorinated Biphenyls (PCBs)

Introduction

Polychlorinated biphenyls (PCBs) are persistent environmental contaminants and bioaccumulate in the food chains, thus posing a health hazard to animals and humans (Safe 1994; Silberhorn *et al*. 1990). There are 209 possible congeners of PCBs, the toxic effects and physical properties of a PCB congener are dependent on the molecular structure. Coplanar PCBs are PCB congeners that are substituted in both *para* and at least two *meta* positions but not in any of the *ortho* positions, they may form a coplanar configuration similar to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and bind strongly to Ah receptor. The toxic effects of coplanar PCBs include immunological and reproductive effects, hepatic toxicities such as hepatimegaly, induction of cytochrome P4501A1 and 1A2 (CYP1A1, CYP1A2). On the other hand, PCBs with chlorine substitutes in two *ortho*-positions are non-coplanar, have low affinity to Ah receptor; they induce the cytochrome P450 2B1 and 2B2 (CYP2B1, CYP2B2), and some of them are neurotoxic (Fischer *et al*. 1998). Previous studies showed that PCB mixtures or individual congeners can act as tumor promoters (Glauert 2001; Silberhorn *et al*. 1990), but the exact mechanisms are still unclear.

The development of liver carcinogenesis process can be divided into at least three consecutive phases: initiation, promotion, and progression (Dragan *et al*. 1993). The first step, initiation, results from a genetic change, which could be due to a chemical, physical, or microbial agent. The genetic alteration is irreversible; however, the initiated phenotype is not fully expressed unless the cell is promoted, which is the second step of carcinogenesis. The promotion stage involves a reversible clonal expansion of single initiated cells. A fraction of hepatic focal lesions represent the precursor to hepatic cancer in the evolution of hepatocellular carcinoma. Progression is the final stage of carcinogenesis and occurs when a neoplasm develops into a benign or a malignant neoplasm. Tumor promoters are a group of compounds that can enhance the growth of hepatic focal lesions by changing cell proliferation and cell death. Tumor promoters, such

as phenobarbital and peroxisome proliferators, do not interact directly with genomic DNA, but chronic administrations of these compounds cause the increased incidence of hepatic tumors (Bayly *et al*. 1994; Cunningham 1996; Gill *et al*. 1998; Roberts *et al*. 1995; Shane *et al*. 2000). The exact mechanisms by which tumor promoters exert their promoting activities are still unknown. Mechanisms have been proposed for the change in growth of hepatic focal lesion caused by tumor promoters, including the inhibition of apoptosis and induction of cell proliferation

The formation of reactive oxygen species (ROS) and is one of mechanisms by which tumor promoters may promote hepatic tumors. Several studies have shown that PCBs can cause oxidative damages, in forms of lipid peroxidation (Kamohara *et al*. 1984; Pelissier *et al*. 1990; Saito 1990), modulation of antioxidants status and antioxidant enzymes (Peltola *et al*. 1994; Twaroski *et al*. 2001b), and oxidative DNA damages (Srinivasan *et al*. 2001). Oxidative stress caused by tumor promoters can alter gene expression. For example, the transcription factor NF-κB has been shown to be activated by PCBs (Tharappel *et al*. 2002). Vitamin E, the major lipophilic chain-breaking antioxidant, is thought to act as a chemopreventive agent at the initiation and the promotion stage. By blocking nitrosamine formation (Ohshima *et al*. 1982) or by inhibiting DNA injury (McVean & Liebler 1999), vitamin E can block initiation, and vitamin E may inhibit promotion by acting on the immune system or by decreasing cell proliferation (Kishimoto *et al*. 1998; van Poppel & van den Berg 1997)

In this study, the effect of dietary α-tocopherol acetate on PCBs-induced hepatic focal lesion growth in diethylnitrosamine (DEN)-initiated Sprague-Dowley rats was investigated. The PCBs used in this study were 3,3',4,4'-tetrachlorobiphenyl (PCB-77), a coplanar PCB, and 2,2'4,4',5,5'-hexachlorobiphenyl (PCB-153), a non-coplanar PCB. Rats were fed purified diet containing different levels of α-tocopherol acetate (10, 50, or 250 ppm) and administrated four doses of corn oil, PCB-77, or PCB-153 after an initiating dose of DEN. The hepatic DNA binding activity of NF-κB was determined. The rates of cell proliferation were measured, both in normal hepatocytes and in placental glutathione S-transferase (PGST)-positive cells.

Materials and methods

Materials: 3,3',4,4'–tetrachlorobiphenyl (PCB-77) and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB-153) were synthesized and characterized as described previously (Schramm *et al*. 1985). Tocopherol-stripped corn oil was from Acros Organics (Morris Plains, NJ). All dry constituents of the purified diet were from Teklad Test Diets (Madison, WI). Alzet osmotic pumps (model 2ML1) were from Alza Scientific Products, Palo Alto, CA. The anti 5-bromo-2'-deoxyuridine (BrdU) antibody was purchased from Bection-Dickinson (San Jose, CA). The anti-placental glutathione S-transferase (PGST) antibody was purchased from Novocastra Laboratories Ltd. (New Castleupon Tyne, England). The Vectastain staining kit was from Vector Laboratories (Burlingame, CA). The α-tocopherol acetate and other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Experimental design and animal treatment: Eighty-one female Sprague-Dawley rats (200 grams) were obtained from Harlan Sprague Dawley (Indianapolis, IN) and housed three rats per cage in a temperature and light-controlled room. The rats were allowed to acclimatize for one week before starting the experiment. After one week, the rats were initiated with diethylnitrosamine (DEN, 150 mg/kg) and were divided into 3 groups (27 rats per group) and placed on a purified diet (see Table 1) containing 10, 50, or 250 ppm vitamin E (α-tocopherol acetate). The rats received the diet *ad libitum*. After one week on the purified diet, rats received an intraperitoneally (i.p.) injection of corn oil, PCB-77, or PCB-153. The rats received another 3 i.p. injections every two weeks, and were euthanized 10 days after the last injection. Alzet osmotic pumps (containing 20 mg/ml BrdU) were surgically implanted in each rat two days before euthanasia. Liver pieces were removed and fixed in 10% formalin, the rest of the liver was frozen in liquid nitrogen and stored in –80 °C until time of assay.

Table 4.1. Composition of purified diets

Constituents	percent of diet
Casein (vitamin free)	14.0
Corn starch	46.57
Dextrose monohydrate	25.5
Cellulose fiber	5.0
Corn oil (tocopherol stripped)	4.0
AIN mineral mix	3.5
L-cystine	0.18
Choline bitartrate	0.25
AIN vitamin mix without vitamin E	1.0
a-tocopheryl acetate	10, 50, or 250 ppm

Measurement of α-tocopherol:

Principle: Tocopherols were extracted from liver homogenates with hexane, and measured using fluorescence detector in a HPLC system.

Chemicals:

1. 100% Ethanol
2. Hydrochloric acid (Fisher Scientific A144-500)
3. Hexane (Fisher Scientific H303-4)
4. Nitrogen Gas
5. Methanol (Mallinckrodt ChromAR HPLC Grade 3041)
6. α-dl-tocopherol standard (Lancaster Synthesis 9231)

Other supplies:

7. Gelman Acrodisc 13 mM with 0.2 μM nylon membrane (Gelman Sciences 4427)
8. HPLC vials for autoinjector (Fisher Scientific 03-375-3E)
9. Inserts for HPLC vials (Sun Brokers 200-232, this is only needed if the sample volume is less than 300 μl)
10. Pyrex glass tubes 9826

11. 0.22 μM Nycaflo nylon membrane filters – Gelman Sciences 66602

Sample preparation:

1. The frozen liver tissues were homogenized with 3-fold volume of 1.15% KCl with 0.1 mM EDTA, pH 7.4. The tissues were placed in a 15 ml tube and homogenized for 20 seconds at 60 output using an Ultra-Turrax homogenizer (Tekmar Co, Cincinnati, OH). The homogenates were aliquoted and kept at –80 °C freezer.
2. 300 μl aliquot was removed from –80 °C freezer and placed in ice.
3. The following were added to a screw cap Pyrex tube:

   1.2 ml 95% Ethanol

   60 μl 0.1 N HCl

   3.6 ml hexane

   300 μl homogenate
4. Each tube was vortexed for 20 seconds using Vortex Genie, then all samples were vortexed for 5 minutes.
5. The samples were spun at 1500 rpm for 5 minutes using Mistral 3000i centrifuge.
6. 3 ml of the top layer (hexane) was transferred to clean glass tube.
7. The samples were dried under a stream of nitrogen gas for 35-45 minutes. The dried samples were kept overnight at –20 °C and resuspended the following day in 320 μl methanol.
8. The α-tocopherol standards were prepared fresh. Stock solution for α-tocopherol (1 mg/ml) was prepared in 100% methanol. The standards with concentration of 0, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, and 50.0 μg/ml were prepared from stock solution.
9. Once the samples were resuspended in methanol and the standards had been prepared, all samples and standards were filtered through 0.2 μM nylon acrodisc Gelman filters. Since the total volume was < 300 μl, small, glass inserts (Sun Brokers) were inserted in the vials to ensure that the autoinjector of the HPLC could easily attain the sample.

Mobile phase and flow rate:

100% HPLC grade methanol – filtered under vacuum using a 0.22 μM Nycaflo nylon membrane. The mobile phase was degased for 20 minutes under vacuum after filtration. The flow rate of the mobile phase in the HPLC was 1 ml per minute.

HPLC analysis:

1. The HPLC system included Shimadzu Systems Controller SCL-10A, Shimadzu Liquid Chromatograph (pump) LC-10AS, Shimadzu Spectrofluorometric Detector RF-551, Shimadzu UV Detector SPD-10A, Shimadzu Auto Injector SIL-10A, Shimadzu Chromatopac CR501, and Column 25 cm x 4.6 mm packed with 5 μm particle size C_{18} material.
2. Fluorometric detector:

   α-tocopherol was measured with fluorometric detector. The excitation was 205 nm and the emission was 350 nm.
3. 50 μl of sample and standard were injected into the HPLC.

Calculations:

$$\alpha\text{-tocopherol } (\mu g/g \text{ liver}) = \frac{\left\{ \begin{array}{l} \alpha\text{ -tocopherol } \mu g/ml \\ \text{(based on linear regression)} \end{array} \right. \quad X \quad 0.32 \text{ ml} \quad X \left(\frac{3.6 \text{ ml}}{3.0 \text{ ml}} \right) \left. \right\}}{\text{g liver in } 300\mu l \text{ of original WLH}}$$

Isolation of nuclear extract:

This procedure was performed as described in chapter 2.

Electrophoretic mobility shift assay (EMSA):

This assay was preformed as described in chapter 2.

Western blotting:

The Western blotting to detect IKKs and IκBs were same as described in chapter 3 with minor modification: equal amount of protein were taken from each sample in a same group and mixed well. The pooled samples were used in Western blotting.

BrdU and placental glutathione S-transferase i (PGSTi) immunohistostaining:

The double immunostaining was carried out on the paraffin sections of liver. The staining procedure was same as described in chapter 2 until step 20, the rest procedure was performed as:

1. The slides were kept in normal goat serum (3 drops of stock normal goat serum mixed with 10 ml of 1 x automation buffer) at 37 C for 10 minutes.
2. Excess serum was drained from slides and Avidin Block was applied to cover section, the slides were incubated for 15 minutes.
3. The slides were dipped briefly in PBS, then covered with Biotin Block for 15 minutes.
4. The slides were drained and followed by covered with GSTpi antibody (the primary antibody was diluted in 40 fold of 1 x automation buffer). The slides were incubated in 37 °C for 30 minutes.
5. The slides were rinsed in PBS for 2 changes, 5 minutes for each change.
6. The biotinylated anti-rabbit IgG (1 drop of IgG was mixed with 3 drops of normal goat serum and 10 ml 1 x automation buffer) was applied to each slide, the slides were incubated at 37 °C for 15 minutes.
7. The slides were rinsed in PBS for 2 changes, 5 minutes for each change.
8. The slides were covered with ABC-AP (2 drops of A, 2 drops and 10 ml of 1 x automation buffer were mixed well, 2 drops each section). The slides were incubated at 37 °C for 15 minutes.
9. The slides were rinsed in PBS for 2 changes, 5 minutes for each change.
10. The slides were stained with Vector Red staining mix for 20 to 30 minutes. [The staining mix was made according to protocol from the vendor: for 5 ml 100 mM Tris-HCl (pH8.2-8.5), 2 drops of reagent 1, 1 drop of levamisole, 2 drops of reagent 2 and 2 drops of reagent 3 were added and mixed well].
11. The slides were monitored under a light microscopy to obtain the best staining.
12. The slides were rinsed in assay buffer for 5 minutes.
13. The slides were rinsed gently in tap water.
14. The hemotoxylin counterstaining and the mounting were performed as described in chapter 2.

Counting of BrdU-stained nuclei:

1. Cells that had incorporated BrdU were easily identified with brown nuclei. All hepatocytes in the PGST-positive foci were counted. For the non-foci area, at least 3000 hepatocellular nuclei were counted randomly per slide.
2. The labeling index was expressed as the percentage of number of labeled nuclei out of the total number of counted nuclei.

Quantitation of altered hepatic foci:

The number and volume of PGST-positive foci were measured using a computer digitizing system; the number of foci/cm^3 (Saltykov method), foci/liver (Saltykov method), the mean focal volume (Saltykov method), and the volume fraction (Delesse method) were analyzed (Campbell *et al*. 1982; Campbell *et al*. 1986; Glauert 1991; Tharappel *et al*. 2002; Xu *et al*. 1998).

Statistical analysis:

Results were first analyzed by a two-way analysis of variance. Individual differences between means were determined using the Bonferroni post hoc test. The results were reported as means $\pm$ standard error of mean (SEM). The level of significance was $p \leq 0.05$.

Results

The relative liver weights (as percentage of body weight) were significantly increased in rats treated with PCBs at all levels of vitamin E, with the highest increase in PCB-77 groups (Table 4.2). Dietary vitamin E had no effect on the relative liver weight. The concentration of vitamin E in liver was increased by dietary vitamin E, with the highest level in those receiving PCB-77 (Figure 4.1).

The number and volume of altered hepatic foci were quantified using placental glutathione S-transferase (PGST) as an immunohistochemical marker. The number of PGST-positive foci per liver and per cm^3, as well as the mean focal volume and the total focal volume as a percentage of liver volume, were significantly increased in rats treated with PCB-77; the number of foci was increased 2-3 fold by PCB-153, but this effect was not statistically significant (Table 4.3). There was a slight but insignificant increase in the

number and volume of PGST-positive foci in PCB-77-treated animals by low level (10 ppm) and high level (250 ppm) of dietary vitamin E compared to animals fed with medium level of vitamin E (50 ppm). The number and volume of PGST-positive foci were not changed by dietary vitamin E in rats treated with corn oil or PCB-153.

To measure cell proliferation in normal and PGST-positive hepatocytes, we quantified the labeling indexes after a 2-day infusion of BrdU using an Alzet osmotic pump. In nonfocal cells, PCB-77 significantly increased the BrdU index in rats fed with the low level (10 ppm) and medium level (50 ppm) of vitamin E (Figure 4.2), but not in animals fed with the high level of vitamin E (250 ppm), compared to the corn oil-treated controls. There was a 56% decrease in the labeling index in the PCB-77 group receiving high vitamin E (250 ppm), compared to the PCB-77 group receiving the medium level of vitamin E ($p = 0.01$). The labeling indexes were not significantly different in rats treated with PCB-153 compared to corn oil-treated controls. There was no significant difference among groups receiving any level of vitamin E in corn oil or PCB-153 groups. The labeling index was higher in focal cells than in nonfocal cells. PCB-77 significantly increased the labeling index in PGST-positive cells compared to corn oil-treated controls, the labeling indexes in PCB-77 groups receiving high level of vitamin E was insignificantly less than those receiving low level ($p = 1.00$) or medium level ($p = 1.00$) of vitamin E.

The DNA binding activity of NF-κB was measured with EMSA. In animals treated with PCB-77, there was a significant ($p < 0.05$) increase in the DNA binding activity of NF-κB (Figure 4.3). Rats fed the high level of vitamin E had a decrease in the PCB-77-induced DNA binding activity of NF-κB. In animals treated with PCB-153, the DNA binding activity of NF-κB was not significantly different from corn oil-treated controls. Dietary vitamin E had no effect on the DNA binding activity of NF-κB in corn oil or PCB-153 groups.

The levels of the IKK and IκB proteins were measured by Western blotting (Figure 4.4). There were no differences in the IKKα, IKKβ, and IKKγ proteins among any group receiving corn oil, PCB-77, or PCB-153, and groups receiving any level of dietary vitamin E. The high level of vitamin E (250 ppm) tended to decrease the IκBα and IκBβ proteins. PCB treatments had no effect on any of these proteins.

Table 4.2. Effect of dietary vitamin E and PCBs on body and liver weights

Vitamin E	Treatment	Liver weight	Body weight	Liver wt/Body wt(%)
Low	corn oil	9.90 ± 0.10	271.44 ± 3.88	3.70 ± 0.06
	PCB-77	14.08 ± 0.72*	240.78 ± 5.12*	6.08 ± 0.18*
	PCB-153	12.71 ± 0.58*	268.50 ± 6.24	4.71 ± 0.13*
Medium	corn oil	9.11 ± 0.42	266.22 ± 3.81	3.39 ± 0.12
	PCB-77	14.23 ± 0.48*	238.78 ± 2.99*	5.98 ± 0.13*
	PCB-153	12.70 ± 0.63*	274.75 ± 6.62	4.46 ± 0.11*
High	corn oil	9.90 ± 0.57	275.38 ± 7.81	3.97 ± 0.12
	PCB-77	14.06 ± 0.49*	239.78 ± 5.23*	5.83 ± 0.12*
	PCB-153	12.28 ± 0.34	269.57 ± 5.67	4.66 ± 0.08*

Results are expressed as mean + SEM. Each group contained 9 animals (n = 9). *Values are significantly different from their respective controls treated with corn oil ($p < 0.05$).

Figure 4.1. α-tocopherol concentrations in liver tissues. Each group contained 9 animals (n = 9). Values represent mean ± SEM. *Values are significantly different from respective controls treated with low level vitamin E ($p < 0.05$). #Values are significantly different from corn oil control groups ($p < 0.05$).

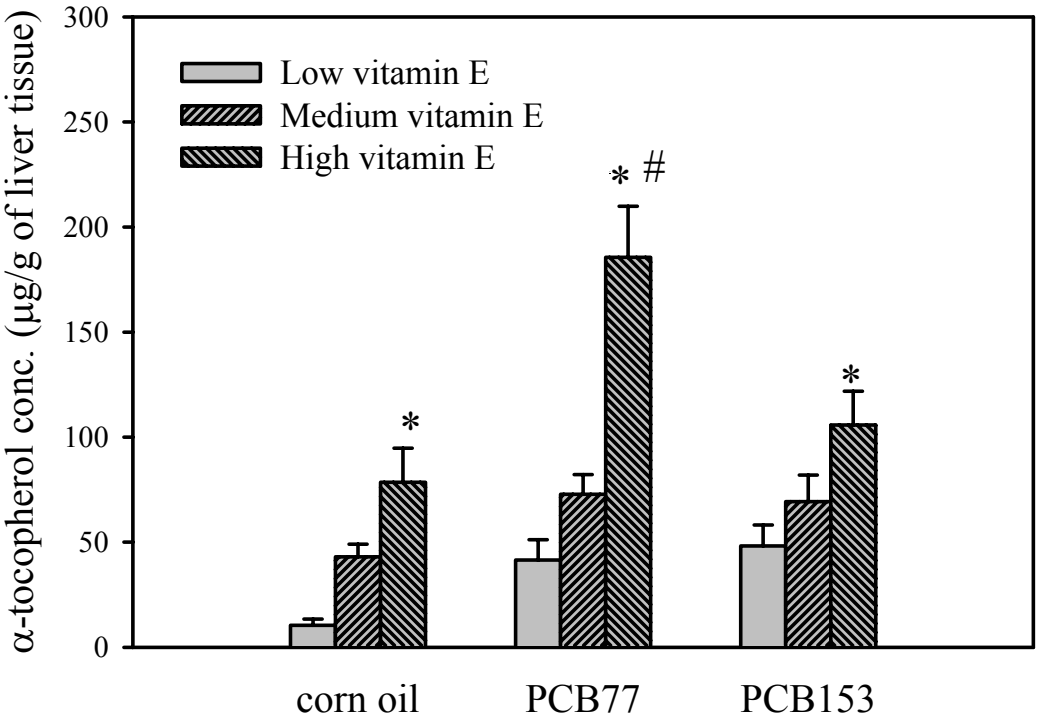

α-tocopherol conc. (μg/g of liver tissue)
300
250
200
150
100
50
0
Low vitamin E
Medium vitamin E
High vitamin E
* #
*
*
corn oil
PCB77
PCB153

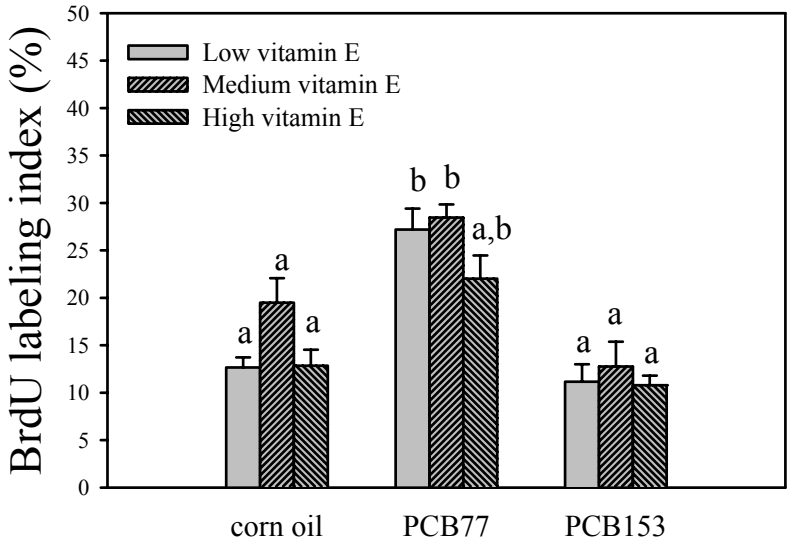
Table 4.3. Effect of vitamin E and PCBs on the induction of PGST-Positive Foci

Vitamin E	Treatment	Foci/cm^3	Foci/liver	Mean focal volume (mm^3 x 10^{-3})	Focal volume (% of liver volume)
Low	corn oil	507 ± 141	5008 ± 1415	3.8 ± 0.4	0.18 ± 0.05
	PCB-77	2120 ± 420*	32250 ± 7011*	125.6 ± 81.6*	13.54 ± 7.16*
	PCB-153	1137 ± 230	14953 ± 3164	5.1 ± 1.0	0.47 ± 0.10
Medium	corn oil	561 ± 96	5256 ± 1101	3.4 ± 0.2	0.21 ± 0.05
	PCB-77	1572 ± 318*	22651 ± 5115*	94.1 ± 49.5*	11.67 ± 6.01*
	PCB-153	934 ± 154	11531 ± 2165	4.5 ± 0.4	0.44 ± 0.07
High	corn oil	516 ± 100	5882 ± 1410	3.9 ± 0.7	0.15 ± 0.04
	PCB-77	2660 ± 946*	37906 ± 13466*	128.0 ± 90.3*	14.60 ± 7.48*
	PCB-153	1161 ± 151	14261 ± 2084	4.8 ± 0.5	0.49 ± 0.06

Results are expressed as mean ± SEM. Each group contained 9 animals (n = 9). *Values are significantly different from their respective controls treated with corn oil ($p < 0.05$).

Figure 4.2. Effect of vitamin E and PCBs on cell proliferation in focal and nonfocal hepatocyte. Rats were administrated BrdU by a 2-day infusion with Alzet osmotic pumps. Tissue sections were immunohistochemically stained for BrdU and labeling indexes were determined in PGST-positive foci and in normal hepatocytes. (A) Labeling indexes in normal hepatocytes. (B) Labeling indexes in PGST-positive hepatocytes. Each group contained 9 animals (n = 9). *Values are significantly different from other groups ($p < 0.05$). a, b, groups with different number are significantly different ($p < 0.05$).

A


BrdU labeling index (%)
50
45
40
35
30
25
20
15
10
5
0
Low vitamin E
Medium vitamin E
High vitamin E
a
a
a
b
b
a,b
a
a
a
corn oil
PCB77
PCB153

B

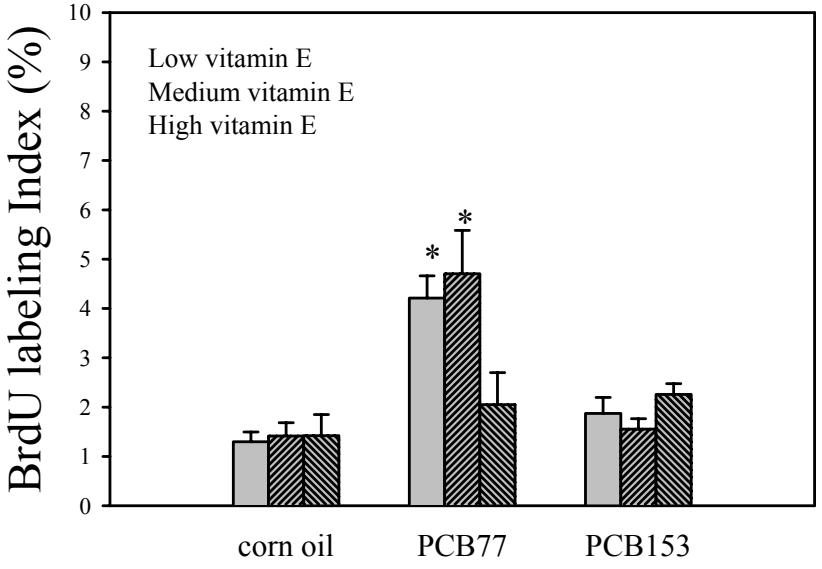
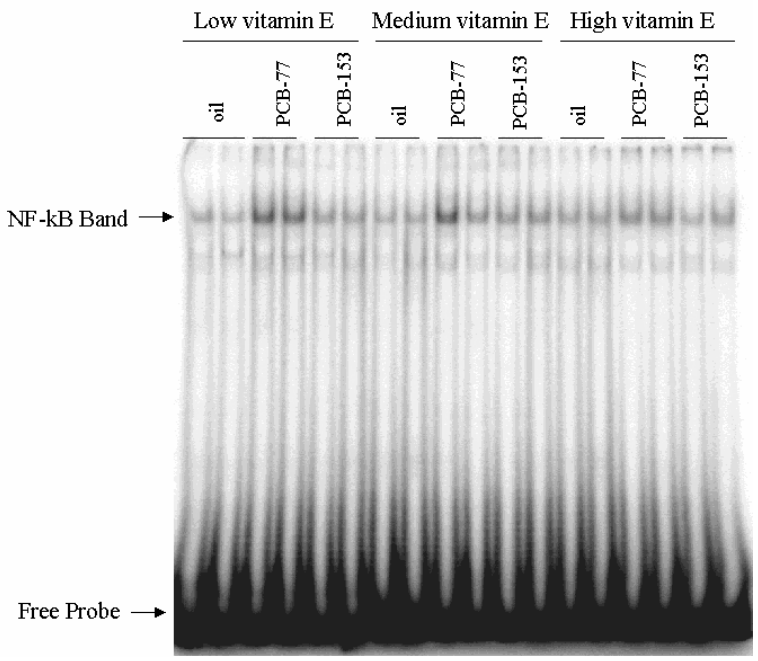

BrdU labeling Index (%)
10
9
8
7
6
5
4
3
2
1
0
Low vitamin E
Medium vitamin E
High vitamin E
*
*
corn oil
PCB77
PCB153

Figure 4.3. Effect of vitamin E and PCBs on the hepatic DNA binding activity of NF-κB. A. Relative NF- κB DNA binding activity was measured by EMSAs using a radiolabeled NF-κB oligonucleotide with liver nuclear extracts from individual rats. Each line contains extract from a single animal (5 μg). B. Quantitation of the NF- κB band as determined by subtracting background counts from the total counts in each NF- κB band. Each group contained nuclear extact from 4 animals (n = 4). Values represent means $\pm$ SEM for 4 animals. *Values are significantly different from their respective control groups treated with corn oil ($p < 0.05$).

A


Low vitamin E
Medium vitamin E
High vitamin E
oil
PCB-77
PCB-153
oil
PCB-77
PCB-153
oil
PCB-77
PCB-153
NF-kB Band
Free Probe

B

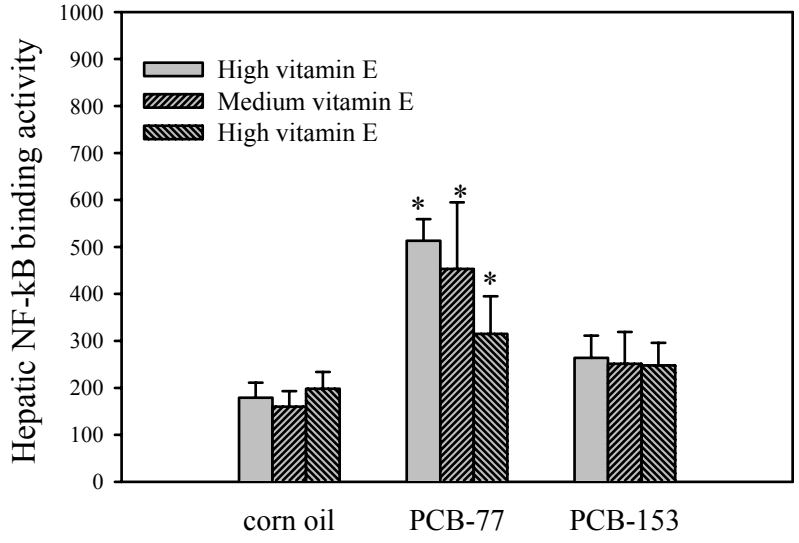
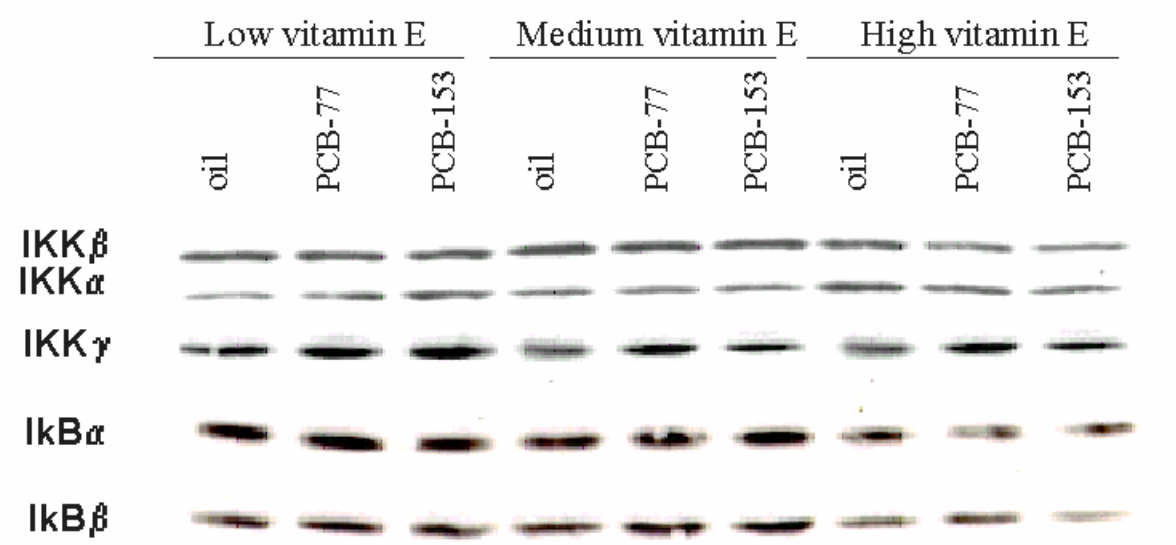

Hepatic NF-kB binding activity
1000
900
800
700
600
500
400
300
200
100
0
High vitamin E
Medium vitamin E
High vitamin E
*
*
*
corn oil
PCB-77
PCB-153

Figure 4.4. Protein levels of cytosolic IκB and IKK. Each line contains equal amount of protein from 3 animals within the same group. 50 μg protein per line.



Discussion

In this study, we tested the hypothesis that oxidative stress induced by PCBs may account for their tumor promoting activities, and therefore that the dietary supplementation of vitamin E would reduce formation of the PCBs-induced altered hepatic focal lesions. The hypothesis that formation of reactive oxygen species accounts for chemically induced hepatocarcinogenesis has been supported by many of the studies (Klaunig & Kamendulis 1999; Klaunig *et al*. 1995; Kolaja & Klaunig 1997). Vitamin E as the major antioxidant in liver has been tested for its role in carcinogenesis. Protective effects of vitamin E have been shown in skin, mammary and esophageal tumors (Burke *et al*. 2000; Hirose *et al*. 1986; Odeleye *et al*. 1992). Liver specific overexpression of the proto-oncogene c-myc and transforming growth factor-α (TGF-α) resulted in increased susceptibility to spontaneous liver tumors (Factor *et al*. 2000); dietary α-tocopherol acetate supplementation (2000 mg/kg diet) decreased intracellular peroxides, chromosomal aberrations, dysplasia and hepatic adenoma incidence in these mice. Vitamin E decreased the hepatic tumor incidence in aflatoxin B_1-treated mice (Nyandieka *et al*. 1990; Nyandieka & Wakhisi 1993; Nyandieka *et al*. 1989). Several studies have showed that increased dietary vitamin E decreased the number of altered hepatic foci or the focal volume with several different protocols (Moore *et al*. 1987) (Hendrich *et al*. 1991; Klaunig & Kamendulis 1999; Kolaja *et al*. 1998; Makpol *et al*. 1997; Ogawa *et al*. 1995; Stevenson *et al*. 1995). However, other studies have indicated an enhancement of carcinogenicity or no effect after vitamin E supplementation (Glauert *et al*. 1990; Kakizaki *et al*. 2001; Kolaja & Klaunig 1997; Lii *et al*. 1999; Lii *et al*. 1997). Kolaja *et al.* have shown that dietary vitamin E supplementation (450 mg/kg diet) for 60 days enhanced the growth of hepatic focal lesions after initiation with DEN (Kolaja & Klaunig 1997). Glauert *et al*. found that dietary supplementation of α-tocopheryl acetate (500 ppm) increased the number of altered hepatic foci and the incidence of tumors in female Sprague-Dawley rats treated with 0.025% ciprofibrate for 21 months (Glauert *et al*. 1990). Studies also indicated no effect of vitamin E supplementation (5000 ppm) on the growth of the γ-glutamyl transpeptidase- and PGST-positive foci in phenobarbital-treated female Sprague-Dawley rats (Lii *et al*. 1999; Lii *et al*. 1997). Vitamin E supplementation showed no protection during DEN-induced hepatocarcinogenesis in transforming growth

factor-alpha (TGF-α) transgenic mice (Kakizaki *et al*. 2001). In the present study, both PCB-77 and PCB-153 increased the number and volume of PGST-positive foci, with PCB-77 exerting the strongest effect. This result is in agreement with previous studies in which hepatic tumor promoting activities of PCB congeners were examined (Dean *et al*. 2002; Glauert *et al*. 1991; Silberhorn *et al*. 1990), including studies using the same protocol (Berberian *et al*. 1995; Tharappel *et al*. 2002). The strong promoting activity of PCB-77 can be explained by the facts that PCB-77 increased cell proliferation, and that the rate of cell proliferation in PGST-positive hepatocytes was higher than in normal cells. This result is in agreement with a previous study using the same protocol (Tharappel *et al*. 2002) and another study in which PCB-77 increased [^{3}H]-thymidine incorporation in an initiation-promotion study using N-nitrosomorpholine as the initiator (Wolfle *et al*. 1988).

PCB-77 increased cell proliferation and NF-κB binding activity in rats fed low (10 ppm) or medium (50 ppm) level of vitamin E. The high level of dietary vitamin E (250 ppm) caused a decrease in cell proliferation as well as in the NF-κB DNA binding activity in PCB-77-treated rat livers; however, the number and volume of PGST-positive foci were slightly increased in the same livers. This conflict can be explained by the different effects in normal cells and in PGST-positive cells: high level of dietary vitamin E significantly decreased the cell proliferation in normal hepatocytes (a 56% reduction, $p = 0.01$), while this decrease was much less in PGST-positive hepatocytes (a 22% reduction, $p = 1.00$), compared to rats receiving medium level of vitamin E; and the rate of cell proliferation in PGST-positive hepatocytes was much higher than that in normal hepatocytes in the same livers (2.05% in normal cells, 22% in PGST-positive cells).

Hepatic concentration of α-tocopherol was affected by the dietary α-tocopherol level; and the level of α-tocopherol was increased by PCBs, with highest increase in rats treated with PCB-77. This increase in hepatic α-tocopherol has been reported earlier (Kato *et al*. 1989; Koremura *et al*. 1990; Twaroski *et al*. 2001a), and proposed as the result of acceleration of α-tocopherol absorption in presence of PCBs (Koremura *et al*. 1990). In our study, pathological examination showed that PCBs administration caused fatty liver, with most severe effect in rats treated with PCB-77, which suggested that the increase of α-tocopherol in liver could be due to the accumulation of fat in livers. PCBs

have been shown to cause multiple effects on lipid metabolism (Azais-Braesco *et al*. 1997; Yamamoto *et al*. 1994), such as hypercholesterolemia, differentiation of stellate cell and increased TBA-reactive substance (TBARS) in liver.

The transcription factor NF-κB has been hypothesized to be important in tumor development (Karin *et al*. 2002) and in the tumor promotion activity of PCBs (Tharappel *et al*. 2002). The hepatic DNA binding activity of NF-κB was increased 1.5 – 2.5 fold by PCB-77, but not by PCB-153. In previous study using the same initiation-promotion protocol, NF-κB was activated more than 4 fold by both PCB-77 and PCB-153 (Tharappel *et al*. 2002). The discrepancy between the two studies could be due to the different type of diets. The level of antioxidants or the activity of the antioxidants could be different. In our study, the vitamin mixture and the α-tocopherol acetate were ordered fresh, and the prepared purified diet was stored at -80°C until the day of use, whereas the chow diet used in the previous study was stored at room temperature until use. The fat source could also be a factor; corn oil has been shown to activate NF-κB in Kupffer cells (Kono *et al*. 2000; Rusyn *et al*. 1999), although corn oil as vehicle did not show any effect on hepatic NF-κB activation in a 2-day study (data not published). The long-term effect of dietary corn oil on hepatic NF-κB activity is still unclear. The purified diet used in our study contained solely corn oil stripped of vitamin E, whereas the fat sources in the unpurified diet used in the previous study were corn, soybean, fish oils and some animal fat (Purina Lab diet 5001). Also, the non-nutritive chemicals in the unpurified diet could be activators or co-activators of NF-κB. Thus, NF-κB seems to be sensitive to dietary factors when exposed to xenobiotics. Animals fed with lowest vitamin E (10 ppm) had the highest activity of NF-κB, while vitamin E supplementation decreased the NF-κB DNA binding activity, which has been shown by many other studies (Nakamura *et al*. 1998; Slim *et al*. 1999). Many investigators believed that NF-κB is involved in tumor promotion because of its role in the regulation of cell cycle and anti-apoptotic process (Karin *et al*. 2002; Mayo & Baldwin 2000). Our study indicated that there was not a correlation between altered hepatic foci induction and NF-κB activation or cell proliferation in animals treated with PCB-77.

Chapter 5. Summary, conclusions, and future studies

Summary and conclusions

In the first study, we tested the hypothesis that PCBs can activate hepatic NF-κB and increase cell proliferation in rats. We used two PCB congeners in this study, the coplanar PCB-77 and the non-coplanar PCB-153. Our results showed that PCB-153 caused a transient increase in hepatic NF-κB DNA binding activity and cell proliferation, with the most significant effect at 2 days after a single dose of PCB-153. But PCB-77 did not show any effect on either NF-κB DNA binding activity or cell proliferation. No synergistic effect on the activation of NF-κB was observed between the two PCBs, but there was a synergistic effect on hepatocyte proliferation between PCB-77 and PCB-153.

To understand the possible relationship between PCB-153-induced NF-κB DNA binding activity and cell proliferation and apoptosis, we used a mouse model that was deficient in the p50 subunit of NF-κB (p50-/-). In this study, a single dose of PCB-153 increased hepatic NF-κB DNA binding activity and the rate of cell proliferation in wild type mice, but not in the p50-/- mice; the longer-term treatment with higher dose of PCB-153 increased cell proliferation in p50-/- livers, but this increase was still less than that in the wild type mice. Usually the rate of apoptosis in livers of adult mice is very low, but p50-/- livers had more "spontaneous" apoptosis, and PCB-153 inhibited apoptosis in the p50-/- livers. Further studies on specific genes that could be involved in the regulation of cell cycle or apoptosis indicated a post-transcriptional regulation of cyclin D1 in the p50-/- livers, based on the observation that p50-/- livers had less cyclin D1 protein than wild type, but the mRNA level of cyclin D1 was same in p50-/- and wild type mice. Studies on the Bcl-x_L proteins showed that there was no difference between p50-/- livers and livers of wild type mice, and that Bcl-x_L protein was not changed by PCB-153 treatment.

In the third study, we tested the hypothesis that the oxidative stress after PCBs exposure accounts for the tumor promoting activity of PCBs, and therefore that dietary supplementation of antioxidant would inhibit the PCBs-induced tumor promotion. The female rats were first initiated with a single dose of DEN, and then received 4 i.p. injections of corn oil, PCB-77 or PCB-153 (300 μmol/kg body weight, once every two

weeks). The rats were fed diets containing different levels of α-tocopherol acetate (10, 50, or 250 ppm) during the promotion period. The placental glutathione *S*-transferase (PGST)-positive foci were used to quantify the preneoplastic lesions; both PCBs increased the number and volume of PGST-positive foci, with PCB-77 showing higher tumor promoting activity. PCB-77 caused an increase in hepatic NF-κB DNA binding activity and hepatocyte proliferation, and this increase was decreased by dietary supplementation of vitamin E, but the number and volume of PGST-positive foci were slightly, though insignificantly, increased in the same animals. The apparent conflict between the growth of altered hepatic focal lesions and the cell proliferation could be explained by the different effects in normal cells and in PGST-positive cells: the high level vitamin E significantly inhibited PCB-77-induced cell proliferation in normal hepatocytes, while this inhibitory effect was much less in the PGST-positive hepatocytes.

Overall, our studies have shown that a non-coplanar PCB can cause an increase in hepatic NF-κB DNA binding activity in rats and mice, and this increase in NF-κB activity contributes to the changes in cell proliferation and apoptosis. Dietary vitamin E supplementation did not show protective effects on the formation of altered hepatic foci (AHF) that were promoted by PCBs, although high level of vitamin E decreased PCBs-induced hepatic NF-κB activation and cell proliferation.

Future studies:

In the initiation-promotion study, the hepatic DNA binding activity of NF-κB was increased 1.5 – 2.5 fold by PCB-77, but not by PCB-153. In previous study using the same initiation-promotion protocol, NF-κB was activated more than 4 fold by both PCB-77 and PCB-153 (Tharappel *et al*. 2002). The discrepancy between the two studies could be due to the different type of diets. The level of antioxidants or the activity of the antioxidants could be different. In our study, the vitamin mixture and the α-tocopherol acetate were ordered fresh, and the prepared purified diet was stored at -80°C until the day of use, whereas the chow diet used in the previous study was stored at room temperature until use. Also, the non-nutritive chemicals in the unpurified diet could be activators or co-activators of NF-κB. Thus, NF-κB seems to be sensitive to dietary factors when exposed to xenobiotics. A study in our laboratory is underway to investigate

the differences of hepatocarcinogens on tumor promotion indices in rats receiving unrefined versus purified diets.

Interactions among different types of cells in liver when exposed to xenobiotics have drawn much attention. Peroxisome proliferators activate Kupffer cells (Rose *et al*. 2000); the activated Kupffer cells then synthesize and secrete TNF-α or other cytokines, which stimulate neighboring hepatocytes to proliferate (Bojes *et al*. 1997). Inactivation of Kupffer cells with dietary glycine or methylpalmitate prevented peroxisome proliferator-induced cell proliferation in rats (Rose *et al*. 1997a; Rose *et al*. 1997b). TNF-α mRNA was increased by WY-14,643 treatment in rats (Bojes *et al*. 1997; Rose *et al*. 1997b). Treatment with anti-TNF-α antibody also prevented WY-14,643-induced cell proliferation (Bojes *et al*. 1997). However, recent studies using TNF-α knockout mice or TNF-α receptor knockout mice have shown that TNF-α is not required for WY14,643-induced hepatocyte proliferation (Anderson *et al*. 2001; Lawrence *et al*. 2001). Van Ess *et al*. recently showed that the TNF-α receptor is essential to the activation of NF-κB by phenobarbital in mice livers because of the fact that phenobarbital caused an increase in hepatic NF-κB DNA binding activity in wild type mice, but not in TNF ($p55^{-/-}/p75^{-/-}$) double receptor knockout mice (Van Ess *et al*. 2002). To our knowledge, no one has investigated the role of Kupffer cells in mitogenesis by PCBs. Whether Kupffer cells or endogenous TNF-α are involved in PCB-induced hepatocellular proliferation is unclear. Studies to answer this question are underway in our laboratory: a short-term study performed on hepatocyte proliferation in rats fed 5% glycine and treated with PCB-153 to measure hepatocyte proliferation in the absence of Kupffer cell function; isolated rat Kupffer cells are treated with PCB-153 to see if they have increased NF-κB DNA binding activity or if they release cytokines in the media. Another study could be done to investigate the necessity of TNF-α pathway in PCB-153-induced hepatocyte proliferation: a short-term study measuring hepatocyte proliferation and NF-κB activation in TNF double receptor knockout mice treated with PCB-153.

Our study showed that p50-/- livers had less cyclin D1 protein than wild type, although they had same level of cylcin D1 mRNA. Assay using [^{35}S]-methionine pulse labeling should be performed, if applicable, to elucidate whether this decreased protein is

due to translational down-regulation or decreased protein stability, or both. Cyclin D1 turnover is governed by ubiquitination and proteasomal degradation, which are positively regulated by glycogen synthase kinase-3β (GSK-3β)-dependent phosphorylation on Threonine-286 (Diehl *et al*. 1998). Recent studies have indicated a crosstalk between GSK-3β and NF-κB. GSK-3β function has been shown to be required for NF-κB-mediated anti-apoptotic response to TNF-α (Hoeflich *et al*. 2000). Disruption of the mouse GSK-3β gene causes severe liver degeneration during mid-gestation (Hoeflich *et al*. 2000), as observed in mice deficient in genes involved in NF-κB activation pathway (Beg *et al*. 1995; Li *et al*. 1999; Rudolph *et al*. 2000; Tanaka *et al*. 1999). The following studies indicated that GSK-3β regulates NF-κB at the level of the transcriptional complex, since the early steps leading to NF-κB activation and NF-κB DNA binding activity were not affected by loss of GSK-3β (Hoeflich *et al*. 2000; Schwabe & Brenner 2002). The mechanism of possible cross-talk between GSK-3β and NF-κB is not clear, but studies have shown that recombinant GSK-3β can phosphorylate the p65 and p105 subunits of NF-κB in cell-free systems (Demarchi *et al*. 2001; Schwabe & Brenner 2002), and that GSK-3β can physically associate with p105 in living cells (Demarchi *et al*. 2001). How disruption of p50 results in decreased cyclin D1 protein as shown in this study need further investigation, and the interaction between NF-κB and GSK-3β is one of the possibilities. To test this, the first study need to be done is to test changes in mRNA and protein levels and the activity of GSK-3β between p50-/- and the wild type mice. If there is more GSK-3β protein or higher activity in the p50-/- livers, this would indicate a mutual regulation between p50 and GSK-3β. To further investigate the mechanism, immortalized cell lines from p50-/- and the wild type mice will be needed, and a functional p50 gene will be introduced into the p50-/- cell and the expression of GSK-3β and cyclin D1 will be checked. In addition, LiCl, the specific inhibitor of GSK-3, has been shown to sensitize primary hepatocytes toward TNF-α-mediated apoptosis by decreasing transcription of NF-κB-dependent inducible nitric oxide synthase (iNOS) gene, but not the NF-κB activation or NF-κB DNA binding activity (Schwabe & Brenner 2002). So LiCl can be used to investigate the effect of deficiency in GSK-3β activity on the cyclin D1 protein turnover. Also, our study showed that PCB-153 failed to induce

NF-κB DNA binding activity in the p50-/- livers, but it inhibited the apoptosis; it is questionable if PCB-153 affects NF-κB at the level of transcriptional complex. If the PCB-153 inhibited LiCl-mediated apoptosis, that would indicate PCB-153 may inhibit apoptosis through regulation of NF-κB transcriptional complex.

The phosphorylation on Threonine-286 by GSK-3β not only mediates cyclin D1 proteasomal degradation, but also promotes cyclin D1 nuclear export (Alt *et al*. 2000; Diehl *et al*. 1998). Cyclin D1 accumulates in the nucleus throughout G1 phase, but it relocates to the cytoplasm during the reminder of interphase (Baldin *et al*. 1993; Diehl *et al*. 1998). Thus, alterations in the intracellular distribution of cyclin D1 during the cell cycle may regulate cyclin D1/CDK4 function (Alt *et al*. 2000; Diehl *et al*. 1998). We examined cyclin D1 protein only in the whole liver homogenates. Thus, an immunohistochemical study using anti-cyclin D1 antibody has to be performed to compare the subcellular localization of cyclin D1 in wild type and p50-/- hepatocytes, and the effect of PCB-153. If there is any significant difference in the rate of cyclin D1 turnover or the subcellular distribution of cyclin D1 between p50-/- livers and wild type, or after PCB-153 treatment, that would explain at least part of mechanisms by which NF-κB may regulate cell proliferation after PCB-153 exposure.

NF-κB is an anti-apoptotic regulator, because the p65 null mice are embryonic lethal due to massive apoptosis in liver (Beg *et al*. 1995). In this study, p50-/- livers had more apoptosis than wild type, which also indicates the anti-apoptotic role of NF-κB. NF-κB regulates expression of certain anti-apoptotic genes, such as cIAP1, cIAP2 (Wang *et al*. 1998), TNF receptor-associated protein 1 and 2 (TRAF1, 2) (Wang *et al*. 1998), and Bcl-2/Bcl-x_L (Lee *et al*. 1999; Tamatani *et al*. 1999; Wang *et al*. 1999). In this study, the protein level of Bcl-x_L was not changed by PCB-153 in both 2-day and 21-day studies, and p50-/- livers had same level of Bcl-x_L as the wild type. Which gene(s) is involved in the increased apoptosis in p50-/- livers is not clear in this study. A study using Affymetrix microarray is underway in our laboratory to compare the change in RNA level of genes in p50-/- and wild type mice in response to PCB-153 treatment; results from this microarray study may indicate genes that may be involved in the changes of cell proliferation and apoptosis by PCB-153 treatment.

Last, the role of NF-κB activation in hepatic tumor promotion by PCBs needs to be determined. Our data have shown that in livers deficient in the p50 subunit of NF-κB, the cell proliferation was not increased by PCB-153 in the short-term (2 days) study, and was increased in the longer-term (21 days) study by higher dose of PCB-153, but this increase was less than that observed in wild type mice. Alteration of cell proliferation and apoptosis has been proposed to be one of the mechanisms of carcinogenesis by tumor promoters. Despite all the data available, it is still a question whether the deficiency in p50 subunit of NF-κB can be protective in PCBs-induced tumorigenesis. To answer this question, an initiation-promotion study using p50-/- mice needs to be done. A PCB mixture and a specific congener have been shown to have liver tumor promoting activity in mice: Anderson *et al*. have shown that PCB mixture Aroclor 1254 increased the incidence of liver adenomas in Swiss NIH:NCr mice initiated with *N*-nitrosodimethylamine (NDMA) (Anderson *et al*. 1994); Kobush *et al*. have shown that PCB-77 treatment in *N*-nitrosomorpholine (NNM)-initiated B6C3F1 mice led to a decrease in the number of G6Pase-positive and –negative foci (Kobusch *et al*. 1989). On the other hand, the volume and the mean diameter of the G6Pase-positive and –negative foci were increased by PCB-77. Therefore, p50-/- mice could be used in an initiation-promotion study to examine the formation of altered hepatic foci or liver tumors with PCBs treatment. If the p50 -/- mice develop fewer hepatic tumors or AHF, we could confirm the protective effect of the deletion of p50 subunit in hepatic carcinogenesis by PCBs.

References

Abate, C., L. Patel, F. J. Rauscher, 3rd & T. Curran: Redox regulation of fos and jun DNA-binding activity in vitro. *Science* 1990, **249,** 1157-61.

Adcock, I. M., C. R. Brown, C. M. Gelder, H. Shirasaki, M. J. Peters & P. J. Barnes: Effects of glucocorticoids on transcription factor activation in human peripheral blood mononuclear cells. *Am J Physiol* 1995, **268,** C331-8.

Albrecht, J. H., M. Y. Hu & F. B. Cerra: Distinct patterns of cyclin D1 regulation in models of liver regeneration and human liver. *Biochem Biophys Res Commun* 1995, **209,** 648-55.

Alt, J. R., J. L. Cleveland, M. Hannink & J. A. Diehl: Phosphorylation-dependent regulation of cyclin D1 nuclear export and cyclin D1-dependent cellular transformation. *Genes Dev* 2000, **14,** 3102-14.

Amstad, P. A., G. Krupitza & P. A. Cerutti: Mechanism of c-fos induction by active oxygen. *Cancer Res* 1992, **52,** 3952-60.

Anderson, L. M., L. E. Beebe, S. D. Fox, H. J. Issaq & R. M. Kovatch: Promotion of mouse lung tumors by bioaccumulated polychlorinated aromatic hydrocarbons. *Exp Lung Res* 1991, **17,** 455-71.

Anderson, L. M., D. Logsdon, S. Ruskie, S. D. Fox, H. J. Issaq, R. M. Kovatch & C. M. Riggs: Promotion by polychlorinated biphenyls of lung and liver tumors in mice. *Carcinogenesis* 1994, **15,** 2245-8.

Anderson, L. M., K. van Havere & J. M. Budinger: Effects of polychlorinated biphenyls on lung and liver tumors initiated in suckling mice by N-nitrosodimethylamine. *J Natl Cancer Inst* 1983, **71,** 157-63.

Anderson, L. M., J. M. Ward, S. D. Fox, H. J. Isaaq & C. W. Riggs: Effects of a single dose of polychlorinated biphenyls to infant mice on N-nitrosodimethylamine-initiated lung and liver tumors. *Int J Cancer* 1986, **38,** 109-16.

Anderson, S. P., C. S. Dunn, R. C. Cattley & J. C. Corton: Hepatocellular proliferation in response to a peroxisome proliferator does not require TNFalpha signaling. *Carcinogenesis* 2001, **22,** 1843-51.

Arcaro, K. F., D. D. Vakharia, Y. Yang & J. F. Gierthy: Lack of synergy by mixtures of weakly estrogenic hydroxylated polychlorinated biphenyls and pesticides. *Environ Health Perspect* 1998, **106 Suppl 4,** 1041-6. 1046arcaro/arcaro-full.html.

Arcaro, K. F., L. Yi, R. F. Seegal, D. D. Vakharia, Y. Yang, D. C. Spink, K. Brosch & J. F. Gierthy: 2,2',6,6'-Tetrachlorobiphenyl is estrogenic in vitro and in vivo. *J Cell Biochem* 1999, **72,** 94-102.

Aronson, K. J., A. B. Miller, C. G. Woolcott, E. E. Sterns, D. R. McCready, L. A. Lickley, E. B. Fish, G. Y. Hiraki, C. Holloway, T. Ross, W. M. Hanna, S. K. SenGupta & J. P. Weber: Breast adipose tissue concentrations of polychlorinated biphenyls and other organochlorines and breast cancer risk. *Cancer Epidemiol Biomarkers Prev* 2000, **9,** 55-63.

Auphan, N., J. A. DiDonato, C. Rosette, A. Helmberg & M. Karin: Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science* 1995, **270,** 286-90.

Awad, M. M. & P. A. Gruppuso: Cell cycle control during liver development in the rat: evidence indicating a role for cyclin D1 posttranscriptional regulation. *Cell Growth Differ* 2000, **11,** 325-34.

Azais-Braesco, V., M. L. Hautekeete, I. Dodeman & A. Geerts: Morphology of liver stellate cells and liver vitamin A content in 3,4,3',4'-tetrachlorobiphenyl-treated rats. *J Hepatol* 1997, **27,** 545-53.

Baldin, V., J. Lukas, M. J. Marcote, M. Pagano & G. Draetta: Cyclin D1 is a nuclear protein required for cell cycle progression in G1. *Genes Dev* 1993, **7,** 812-21.

Bartek J, B. J., Lukas J.: The retinoblastoma protein pathway and the restriction point. *Curr Opin Cell Biol* 1996, **8,** 805-14.

Basu, A. K. & L. J. Marnett: Molecular requirements for the mutagenicity of malondialdehyde and related acroleins. *Cancer Res* 1984, **44,** 2848-54.

Bayly, A. C., R. A. Roberts & C. Dive: Suppression of liver cell apoptosis in vitro by the non-genotoxic hepatocarcinogen and peroxisome proliferator nafenopin. *J Cell Biol* 1994, **125,** 197-203.

Beebe, L. E., Y. E. Kim, S. Amin, C. W. Riggs, R. M. Kovatch & L. M. Anderson: Comparison of transplacental and neonatal initiation of mouse lung and liver

tumors by N-nitrosodimethylamine (NDMA) and 4-(methylnitrosamino)- 1-(3-pyridyl)-1-butanone (NNK) and promotability by a polychlorinated biphenyls mixture (Aroclor 1254). *Carcinogenesis* 1993, **14,** 1545-8.

Beg, A., W. Sha, R. Bronson, S. Ghosh & D. Baltimore: Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature* 1995, **376,** 167-70.

Beijersbergen RL, B. R.: Cell cycle regulation by the retinoblastoma family of growth inhibitory proteins. *Biochim Biophys Acta* 1996, **1287,** 103-20.

Benamira, M., K. Johnson, A. Chaudhary, K. Bruner, C. Tibbetts & L. J. Marnett: Induction of mutations by replication of malondialdehyde-modified M13 DNA in Escherichia coli: determination of the extent of DNA modification, genetic requirements for mutagenesis, and types of mutations induced. *Carcinogenesis* 1995, **16,** 93-9.

Berberian, I., L. C. Chen, F. R. Robinson, H. P. Glauert, C. K. Chow & L. W. Robertson: Effect of dietary retinyl palmitate on the promotion of altered hepatic foci by 3,3',4,4'-tetrachlorobiphenyl and 2,2',4,4',5,5'-hexachlorobiphenyl in rats initiated with diethylnitrosamine. *Carcinogenesis* 1995, **16,** 393-8.

Biswas, D. K., A. P. Cruz, E. Gansberger & A. B. Pardee: Epidermal growth factor-induced nuclear factor kappa B activation: A major pathway of cell-cycle progression in estrogen-receptor negative breast cancer cells. *Proc Natl Acad Sci U S A* 2000, **97,** 8542-7.

Bohnenberger, S., B. Wagner, H. J. Schmitz & D. Schrenk: Inhibition of apoptosis in rat hepatocytes treated with 'non-dioxin- like' polychlorinated biphenyls. *Carcinogenesis* 2001, **22,** 1601-6.

Bojes, H. K., D. R. Germolec, P. Simeonova, A. Bruccoleri, R. Schoonhoven, M. I. Luster & R. G. Thurman: Antibodies to tumor necrosis factor alpha prevent increases in cell replication in liver due to the potent peroxisome proliferator, WY- 14,643. *Carcinogenesis* 1997, **18,** 669-74.

Bondy, S. C. & S. Naderi: Contribution of hepatic cytochrome P450 systems to the generation of reactive oxygen species. *Biochem Pharmacol* 1994, **48,** 155-9.

Bonefeld-Jorgensen, E. C., H. R. Andersen, T. H. Rasmussen & A. M. Vinggaard: Effect of highly bioaccumulated polychlorinated biphenyl congeners on estrogen and androgen receptor activity. *Toxicology* 2001, **158,** 141-53.

Booij, K. & B. L. van Drooge: Polychlorinated biphenyls and hexachlorobenzene in atmosphere, sea- surface microlayer, and water measured with semi-permeable membrane devices (SPMDs). *Chemosphere* 2001, **44,** 91-8.

Buchmann, A., W. Kunz, C. R. Wolf, F. Oesch & L. W. Robertson: Polychlorinated biphenyls, classified as either phenobarbital- or 3- methylcholanthrene-type inducers of cytochrome P-450, are both hepatic tumor promoters in diethylnitrosamine-initiated rats. *Cancer Lett* 1986, **32,** 243-53.

Buchmann, A., S. Ziegler, A. Wolf, L. W. Robertson, S. K. Durham & M. Schwarz: Effects of polychlorinated biphenyls in rat liver: correlation between primary subcellular effects and promoting activity. *Toxicol Appl Pharmacol* 1991, **111,** 454-68.

Burke, K. E., J. Clive, G. F. Combs, Jr., J. Commisso, C. L. Keen & R. M. Nakamura: Effects of topical and oral vitamin E on pigmentation and skin cancer induced by ultraviolet irradiation in Skh:2 hairless mice. *Nutr Cancer* 2000, **38,** 87-97.

Campbell, H. A., H. C. Pitot, V. R. Potter & B. A. Laishes: Application of quantitative stereology to the evaluation of enzyme- altered foci in rat liver. *Cancer Res* 1982, **42,** 465-72.

Campbell, H. A., Y. D. Xu, M. H. Hanigan & H. C. Pitot: Application of quantitative stereology to the evaluation of phenotypically heterogeneous enzyme-altered foci in the rat liver. *J Natl Cancer Inst* 1986, **76,** 751-67.

Cerutti, P. A. & B. F. Trump: Inflammation and oxidative stress in carcinogenesis. *Cancer Cells* 1991, **3,** 1-7.

Chevalier, S. & R. A. Roberts: G1-arrested FaO cells re-enter the cell cycle upon stimulation with the rodent non-genotoxic hepatocarcinogen nafenopin. *Carcinogenesis* 1999, **20,** 1209-13.

Christensen, J. G., A. J. Gonzales, R. C. Cattley & T. L. Goldsworthy: Regulation of apoptosis in mouse hepatocytes and alteration of apoptosis by nongenotoxic carcinogens. *Cell Growth Differ* 1998, **9,** 815-25.

Christensen, J. G., E. H. Romach, L. N. Healy, A. J. Gonzales, S. P. Anderson, D. E. Malarkey, J. C. Corton, T. R. Fox, R. C. Cattley & T. L. Goldsworthy: Altered bcl-2 family expression during non-genotoxic hepatocarcinogenesis in mice. *Carcinogenesis* 1999, **20,** 1583-90.

Chung, J., C. Liu, D. E. Smith, H. K. Seitz, R. M. Russell & X. D. Wang: Restoration of retinoic acid concentration suppresses ethanol-enhanced c-Jun expression and hepatocyte proliferation in rat liver. *Carcinogenesis* 2001, **22,** 1213-9.

Colquhoun, A. & R. I. Schumacher: gamma-Linolenic acid and eicosapentaenoic acid induce modifications in mitochondrial metabolism, reactive oxygen species generation, lipid peroxidation and apoptosis in Walker 256 rat carcinosarcoma cells. *Biochim Biophys Acta* 2001, **1533,** 207-19.

Connor, K., K. Ramamoorthy, M. Moore, M. Mustain, I. Chen, S. Safe, T. Zacharewski, B. Gillesby, A. Joyeux & P. Balaguer: Hydroxylated polychlorinated biphenyls (PCBs) as estrogens and antiestrogens: structure-activity relationships. *Toxicol Appl Pharmacol* 1997, **145,** 111-23.

Crawford, D.: Oxidant stress induces the prooncogenes c-fos and c-myc in mouse epidermal cells. *Bull. Cancer* 1990, **77,** 501-502.

Cunningham, M. L.: Role of increased DNA replication in the carcinogenic risk of nonmutagenic chemical carcinogens. *Mutat Res* 1996, **365,** 59-69.

Das, K. C. & C. W. White: **Activation of NF-B by Antineoplastic Agents. ROLE OF PROTEIN KINASE C**. *J. Biol. Chem.* 1997, **272,** 14914-14920.

Davidson, N. E.: Environmental estrogens and breast cancer risk. *Curr Opin Oncol* 1998, **10,** 475-8.

De Bosscher, K., M. L. Schmitz, W. Vanden Berghe, S. Plaisance, W. Fiers & G. Haegeman: Glucocorticoid-mediated repression of nuclear factor-kappaB-dependent transcription involves direct interference with transactivation. *Proc Natl Acad Sci U S A* 1997, **94,** 13504-9.

De Bosscher, K., W. Vanden Berghe, L. Vermeulen, S. Plaisance, E. Boone & G. Haegeman: Glucocorticoids repress NF-kappaB-driven genes by disturbing the interaction of p65 with the basal transcription machinery, irrespective of coactivator levels in the cell. *Proc Natl Acad Sci U S A* 2000, **97,** 3919-24.

De Smaele, E., F. Zazzeroni, S. Papa, D. U. Nguyen, R. Jin, J. Jones, R. Cong & G. Franzoso: Induction of gadd45beta by NF-kappaB downregulates pro-apoptotic JNK signalling. *Nature* 2001, **414,** 308-13.

Dean, C. E., Jr., S. A. Benjamin, L. S. Chubb, J. D. Tessari & T. J. Keefe: Nonadditive hepatic tumor promoting effects by a mixture of two structurally different polychlorinated biphenyls in female rat livers. *Toxicol Sci* 2002, **66,** 54-61.

Deane, N. G., M. A. Parker, R. Aramandla, L. Diehl, W. J. Lee, M. K. Washington, L. B. Nanney, Y. Shyr & R. D. Beauchamp: Hepatocellular carcinoma results from chronic cyclin D1 overexpression in transgenic mice. *Cancer Res* 2001, **61,** 5389-95.

DeAngelis, R., K. Kovalovich, D. Cressman & R. Taub: Normal liver regeneration in p50/nuclear factor kappaB1 knockout mice. *Hepatology* 2001, **33,** 915-24.

Dedon, P. C., J. P. Plastaras, C. A. Rouzer & L. J. Marnett: Indirect mutagenesis by oxidative DNA damage: formation of the pyrimidopurinone adduct of deoxyguanosine by base propenal. *Proc Natl Acad Sci U S A* 1998, **95,** 11113-6.

Dejardin, E., G. Bonizzi, A. Bellahcene, V. Castronovo, M. P. Merville & V. Bours: Highly-expressed p100/p52 (NFKB2) sequesters other NF-kappa B-related proteins in the cytoplasm of human breast cancer cells. *Oncogene* 1995, **11,** 1835-41.

Demarchi, F., R. Verardo, B. Varnum, C. Brancolini & C. Schneider: Gas6 anti-apoptotic signaling requires NF-kappa B activation. *J Biol Chem* 2001, **276,** 31738-44.

Demers, A., P. Ayotte, J. Brisson, S. Dodin, J. Robert & E. Dewailly: Plasma concentrations of polychlorinated biphenyls and the risk of breast cancer: a congener-specific analysis. *Am J Epidemiol* 2002, **155,** 629-35.

Deml, E. & D. Oesterle: Sex-dependent promoting effect of polychlorinated biphenyls on enzyme- altered islands induced by diethylnitrosamine in rat liver. *Carcinogenesis* 1982, **3,** 1449-52.

Deml, E. & D. Oesterle: Dose-response of promotion by polychlorinated biphenyls and chloroform in rat liver foci bioassay. *Arch Toxicol* 1987, **60,** 209-11.

Denk, B., J. G. Filser, E. Deml, W. Kessler, J. Shen & D. Oesterle: Dose-dependent emergence of preneoplastic foci in rat livers after exposure to 2-nitropropane. *Arch Toxicol* 1990, **64,** 329-31.

Diehl, J. A., M. Cheng, M. F. Roussel & C. J. Sherr: Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev* 1998, **12,** 3499-511.

Diwan, B. A., J. M. Ward, Y. Kurata & J. M. Rice: Dissimilar frequency of hepatoblastomas and hepatic cystadenomas and adenocarcinomas arising in hepatocellular neoplasms of D2B6F1 mice initiated with N-nitrosodiethylamine and subsequently given Aroclor- 1254, dichlorodiphenyltrichloroethane, or phenobarbital. *Toxicol Pathol* 1994, **22,** 430-9.

Dogra, S., J. G. Filser, C. Cojocel, H. Greim, U. Regel, F. Oesch & L. W. Robertson: Long-term effects of commercial and congeneric polychlorinated biphenyls on ethane production and malondialdehyde levels, indicators of in vivo lipid peroxidation. *Arch Toxicol* 1988, **62,** 369-74.

Dorgan, J. F., J. W. Brock, N. Rothman, L. L. Needham, R. Miller, H. E. Stephenson, Jr., N. Schussler & P. R. Taylor: Serum organochlorine pesticides and PCBs and breast cancer risk: results from a prospective analysis (USA). *Cancer Causes Control* 1999, **10,** 1-11.

Dragan, Y. P., L. Sargent, Y. D. Xu, Y. H. Xu & H. C. Pitot: The initiation-promotion-progression model of rat hepatocarcinogenesis. *Proc Soc Exp Biol Med* 1993, **202,** 16-24.

Durham, S. K. & A. Brouwer: 3,4,3',4'-tetrachlorobiphenyl-induced effects in the rat liver. I. Serum and hepatic retinoid reduction and morphologic changes. *Toxicol Pathol* 1989, **17,** 536-44.

Factor, V. M., D. Laskowska, M. R. Jensen, J. T. Woitach, N. C. Popescu & S. S. Thorgeirsson: Vitamin E reduces chromosomal damage and inhibits hepatic tumor formation in a transgenic mouse model. *Proc Natl Acad Sci U S A* 2000, **97,** 2196-201.

Fadhel, Z., Z. Lu, L. W. Robertson & H. P. Glauert: Effect of 3,3',4,4'-tetrachlorobiphenyl and 2,2',4,4',5,5'- hexachlorobiphenyl on the induction of

hepatic lipid peroxidation and cytochrome P-450 associated enzyme activities in rats. *Toxicology* 2002, **175,** 15-25.

Fielden, M. R., I. Chen, B. Chittim, S. H. Safe & T. R. Zacharewski: Examination of the estrogenicity of 2,4,6,2',6'-pentachlorobiphenyl (PCB 104), its hydroxylated metabolite 2,4,6,2',6'-pentachloro-4- biphenylol (HO-PCB 104), and a further chlorinated derivative, 2,4,6,2',4',6'-hexachlorobiphenyl (PCB 155). *Environ Health Perspect* 1997, **105,** 1238-48.

Fischer, L. J., R. F. Seegal, P. E. Ganey, I. N. Pessah & P. R. Kodavanti: Symposium overview: toxicity of non-coplanar PCBs. *Toxicol Sci* 1998, **41,** 49-61.

Fromberg, A., M. Cleemann & L. Carlsen: Review on persistent organic pollutants in the environment of Greenland and Faroe Islands. *Chemosphere* 1999, **38,** 3075-93.

Gierthy, J. F., K. F. Arcaro & M. Floyd: Assessment of PCB estrogenicity in a human breast cancer cell line. *Chemosphere* 1997, **34,** 1495-505.

Giesy, J. P., W. W. Bowerman, M. A. Mora, D. A. Verbrugge, R. A. Othoudt, J. L. Newsted, C. L. Summer, R. J. Aulerich, S. J. Bursian, J. P. Ludwig & et al.: Contaminants of fishes from Great Lakes-influenced sections and above dams of three Michigan rivers: III. Implications for health of bald eagles. *Arch Environ Contam Toxicol* 1995, **29,** 309-21.

Giesy, J. P., D. A. Verbrugge, R. A. Othout, W. W. Bowerman, M. A. Mora, P. D. Jones, J. L. Newsted, C. Vandervoort, S. N. Heaton, R. J. Aulerich & et al.: Contaminants in fishes from Great Lakes-influenced sections and above dams of three Michigan rivers. I: Concentrations of organo chlorine insecticides, polychlorinated biphenyls, dioxin equivalents, and mercury. *Arch Environ Contam Toxicol* 1994, **27,** 202-12.

Gill, J. H., N. H. James, R. A. Roberts & C. Dive: The non-genotoxic hepatocarcinogen nafenopin suppresses rodent hepatocyte apoptosis induced by TGFbeta1, DNA damage and Fas. *Carcinogenesis* 1998, **19,** 299-304.

Glauert, H. P.: Histochemical and stereological analysis of putative preneoplastic hepatic lesions. *Prog Histochem Cytochem* 1991, **23,** 84-90.

Glauert, H. P., Robertson, L. W., and Silberhorn, E. M.: PCBs and tumor promotion. *PCBs: Recent Advances in the Environmental Toxicology and Health Effect* 2001**,** 355-371.

Glauert, H. P., M. M. Beaty, T. D. Clark, W. S. Greenwell, V. Tatum, L. C. Chen, T. Borges, T. L. Clark, S. R. Srinivasan & C. K. Chow: Effect of dietary vitamin E on the development of altered hepatic foci and hepatic tumors induced by the peroxisome proliferator ciprofibrate. *J Cancer Res Clin Oncol* 1990, **116,** 351-6.

Glauert, H. P., L. T. Lay, W. S. Kennan & H. C. Pitot: Effect of dietary fat on the initiation of hepatocarcinogenesis by diethylnitrosamine or 2-acetylaminofluorene in rats. *Carcinogenesis* 1991, **12,** 991-5.

Goeptar, A. R., H. Scheerens & N. P. Vermeulen: Oxygen and xenobiotic reductase activities of cytochrome P450. *Crit Rev Toxicol* 1995, **25,** 25-65.

Guo, Y. L., J. J. Ryan, B. P. Lau, M. L. Yu & C. C. Hsu: Blood serum levels of PCBs and PCDFs in Yucheng women 14 years after exposure to a toxic rice oil. *Arch Environ Contam Toxicol* 1997, **33,** 104-8.

Guo, Y. L., M. L. Yu, C. C. Hsu & W. J. Rogan: Chloracne, goiter, arthritis, and anemia after polychlorinated biphenyl poisoning: 14-year follow-Up of the Taiwan Yucheng cohort. *Environ Health Perspect* 1999, **107,** 715-9.

Guttridge, D. C., C. Albanese, J. Y. Reuther, R. G. Pestell & A. S. Baldwin, Jr.: NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1. *Mol Cell Biol* 1999, **19,** 5785-99.

Haag-Gronlund, M., N. Johansson, R. Fransson-Steen, H. Hakansson, G. Scheu & L. Warngard: Interactive effects of three structurally different polychlorinated biphenyls in a rat liver tumor promotion bioassay. *Toxicol Appl Pharmacol* 1998, **152,** 153-65.

Haag-Gronlund, M., Y. Kato, R. Fransson-Steen, G. Scheu & L. Warngard: Promotion of enzyme altered foci in female rat 2,3,3',4,4',5- hexachlorobiphenyl. *Toxicol Appl Pharmacol* 1997a, **147,** 46-55.

Haag-Gronlund, M., L. Warngard, S. Flodstrom, G. Scheu, T. Kronevi, U. G. Ahlborg & R. Fransson-Steen: Promotion of altered hepatic foci by 2,3',4,4',5-

pentachlorobiphenyl in Sprague-Dawley female rats. *Fundam Appl Toxicol* 1997b, **35,** 120-30.

Haag-Gronlund M, C. R., Scheu G, Warngard L, Fransson-Steen R: Analysis of rat liver foci growth with a quantitative two-cell model after treatment with 2,4,5,3',4'-pentachlorobiphenyl. *Toxicol Sci* 2000, **57,** 32-42.

Hansen, J. C.: Environmental contaminants and human health in the Arctic. *Toxicol Lett* 2000, **112-113,** 119-25.

Hassoun, E. A., F. Li, A. Abushaban & S. J. Stohs: Production of superoxide anion, lipid peroxidation and DNA damage in the hepatic and brain tissues of rats after subchronic exposure to mixtures of TCDD and its congeners. *J Appl Toxicol* 2001, **21,** 211-9.

Hassoun, E. A., H. Wang, A. Abushaban & S. J. Stohs: Induction of oxidative stress in the tissues of rats after chronic exposure to TCDD, 2,3,4,7,8-pentachlorodibenzofuran, and 3,3',4,4',5- pentachlorobiphenyl. *J Toxicol Environ Health A* 2002, **65,** 825-42.

Heck, S., K. Bender, M. Kullmann, M. Gottlicher, P. Herrlich & A. C. Cato: I kappaB alpha-independent downregulation of NF-kappaB activity by glucocorticoid receptor. *Embo J* 1997, **16,** 4698-707.

Helzlsouer, K. J., A. J. Alberg, H. Y. Huang, S. C. Hoffman, P. T. Strickland, J. W. Brock, V. W. Burse, L. L. Needham, D. A. Bell, J. A. Lavigne, J. D. Yager & G. W. Comstock: Serum concentrations of organochlorine compounds and the subsequent development of breast cancer. *Cancer Epidemiol Biomarkers Prev* 1999, **8,** 525-32.

Hemming, H., Y. Bager, S. Flodstrom, I. Nordgren, T. Kronevi, U. G. Ahlborg & L. Warngard: Liver tumour promoting activity of 3,4,5,3',4'-pentachlorobiphenyl and its interaction with 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Eur J Pharmacol* 1995, **292,** 241-9.

Hemming, H., S. Flodstrom, L. Warngard, A. Bergman, T. Kronevi, I. Nordgren & U. G. Ahlborg: Relative tumour promoting activity of three polychlorinated biphenyls in rat liver. *Eur J Pharmacol* 1993, **248,** 163-74.

Hendrich, S., P. Duitsman, S. K. Krueger, A. Jackson & R. K. Myers: Effects of alpha-tocopherol, phenobarbital, and butylated hydroxyanisole during promotion of diethylnitrosamine-initiated rat hepatocarcinogenesis. *Nutr Cancer* 1991, **15,** 53-62.

Henry, D. O., S. A. Moskalenko, K. J. Kaur, M. Fu, R. G. Pestell, J. H. Camonis & M. A. White: Ral GTPases contribute to regulation of cyclin D1 through activation of NF-kappaB. *Mol Cell Biol* 2000, **20,** 8084-92.

Hinz, M., D. Krappmann, A. Eichten, A. Heder, C. Scheidereit & M. Strauss: NF-kappaB function in growth control: regulation of cyclin D1 expression and G0/G1-to-S-phase transition. *Mol Cell Biol* 1999, **19,** 2690-2698.

Hirose, M., A. Masuda, T. Inoue, S. Fukushima & N. Ito: Modification by antioxidants and p,p'-diaminodiphenylmethane of 7,12- dimethylbenz[a]anthracene-induced carcinogenesis of the mammary gland and ear duct in CD rats. *Carcinogenesis* 1986, **7,** 1155-9.

Hirose, M., T. Shirai, H. Tsuda, S. Fukushima, T. Ogiso & N. Ito: Effect of phenobarbital, polychlorinated biphenyl and sodium saccharin on hepatic and renal carcinogenesis in unilaterally nephrectomized rats given N-ethyl-N-hydroxyethylnitrosamine orally. *Carcinogenesis* 1981, **2,** 1299-302.

Hoeflich, K. P., J. Luo, E. A. Rubie, M. S. Tsao, O. Jin & J. R. Woodgett: Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. *Nature* 2000, **406,** 86-90.

Holford, T. R., T. Zheng, S. T. Mayne, S. H. Zahm, J. D. Tessari & P. Boyle: Joint effects of nine polychlorinated biphenyl (PCB) congeners on breast cancer risk. *Int J Epidemiol* 2000, **29,** 975-82.

Hotz-Wagenblatt, A. & D. Shalloway: Gap junctional communication and neoplastic transformation. *Crit Rev Oncog* 1993, **4,** 541-58.

Hsieh, S. F., Y. Y. Yen, S. J. Lan, C. C. Hsieh, C. H. Lee & Y. C. Ko: A cohort study on mortality and exposure to polychlorinated biphenyls. *Arch Environ Health* 1996, **51,** 417-24.

Huber, W., B. Kraupp-Grasl, H. Esterbauer & R. Schulte-Hermann: Role of oxidative stress in age dependent hepatocarcinogenesis by the peroxisome proliferator nafenopin in the rat. *Cancer Res* 1991, **51,** 1789-92.

Hunt, C. R., J. E. Sim, S. J. Sullivan, T. Featherstone, W. Golden, C. Von Kapp-Herr, R. A. Hock, R. A. Gomez, A. J. Parsian & D. R. Spitz: Genomic instability and catalase gene amplification induced by chronic exposure to oxidative stress. *Cancer Res* 1998, **58,** 3986-92.

Hunter, D. J., S. E. Hankinson, F. Laden, G. A. Colditz, J. E. Manson, W. C. Willett, F. E. Speizer & M. S. Wolff: Plasma organochlorine levels and the risk of breast cancer. *N Engl J Med* 1997, **337,** 1253-8.

Ikeda, H. & K. Fujiwara: Retinoic acid inhibits DNA and albumin synthesis stimulated by growth factor in adult rat hepatocytes in primary culture. *Biochem Biophys Res Commun* 1993, **191,** 675-80.

Ikeda, M.: Comparison of clinical picture between Yusho/Yucheng cases and occupational PCB poisoning cases. *Chemosphere* 1996, **32,** 559-66.

Ito, N., H. Nagasaki, M. Arai, S. Makiura, S. Sugihara & K. Hirao: Histopathologic studies on liver tumorigenesis induced in mice by technical polychlorinated biphenyls and its promoting effect on liver tumors induced by benzene hexachloride. *J Natl Cancer Inst* 1973, **51,** 1637-46.

Javelaud, D. & F. Besancon: NF-kappa B activation results in rapid inactivation of JNK in TNF alpha- treated Ewing sarcoma cells: a mechanism for the anti-apoptotic effect of NF-kappa B. *Oncogene* 2001, **20,** 4365-72.

Jensen, S., A. G. Johnels, M. Olsson & G. Otterlind: DDT and PCB in marine animals from Swedish waters. *Nature* 1969, **224,** 247-50.

Johansson, M., S. Nilsson & B. O. Lund: Interactions between methylsulfonyl PCBs and the glucocorticoid receptor. *Environ Health Perspect* 1998, **106,** 769-72. 772johansson/johansson-full.html.

Joo, M., Y. K. Kang, M. R. Kim, H. K. Lee & J. J. Jang: Cyclin D1 overexpression in hepatocellular carcinoma. *Liver* 2001, **21,** 89-95.

Joyce, D., B. Bouzahzah, M. Fu, C. Albanese, M. D'Amico, J. Steer, J. U. Klein, R. J. Lee, J. E. Segall, J. K. Westwick, C. J. Der & R. G. Pestell: Integration of Rac-

dependent regulation of cyclin D1 transcription through a nuclear factor-kappaB-dependent pathway. *J Biol Chem* 1999, **274,** 25245-9.

Kakizaki, S., H. Takagi, T. Fukusato, M. Toyoda, N. Horiguchi, K. Sato, H. Takayama, T. Nagamine & M. Mori: Effect of alpha-tocopherol on hepatocarcinogenesis in transforming growth factor-alpha (TGF-alpha) transgenic mice treated with diethylnitrosamine. *Int J Vitam Nutr Res* 2001, **71,** 261-7.

Kaltschmidt, B., C. Kaltschmidt, S. P. Hehner, W. Droge & M. L. Schmitz: Repression of NF-kappaB impairs HeLa cell proliferation by functional interference with cell cycle checkpoint regulators. *Oncogene* 1999, **18,** 3213-25.

Kamohara, K., N. Yagi & Y. Itokawa: Mechanism of lipid peroxide formation in polychlorinated biphenyls (PCB) and dichlorodiphenyltrichloroethane (DDT)-poisoned rats. *Environ Res* 1984, **34,** 18-23.

Karin, M., Y. Cao, F. R. Greten & Z. W. Li: NF-kappaB in cancer: from innocent bystander to major culprit. *Nature Rev Cancer* 2002, **2,** 301-10.

Kato, N., Y. Momota & T. Kusuhara: Changes in distribution of alpha-tocopherol and cholesterol in serum lipoproteins and tissues of rats by dietary PCB and dietary level of protein. *J Nutr Sci Vitaminol (Tokyo)* 1989, **35,** 655-60.

Khoshnan, A., C. Tindell, I. Laux, D. Bae, B. Bennett & A. E. Nel: The NF-kappa B cascade is important in Bcl-xL expression and for the anti-apoptotic effects of the CD28 receptor in primary human CD4+ lymphocytes. *J Immunol* 2000, **165,** 1743-54.

Kikuchi, M.: Autopsy of patients with yusho. *Prog Clin Biol Res* 1984, **137,** 19-30.

Kinoshita, A., H. Wanibuchi, S. Imaoka, M. Ogawa, C. Masuda, K. Morimura, Y. Funae & S. Fukushima: Formation of 8-hydroxydeoxyguanosine and cell-cycle arrest in the rat liver via generation of oxidative stress by phenobarbital: association with expression profiles of p21(WAF1/Cip1), cyclin D1 and Ogg1. *Carcinogenesis* 2002, **23,** 341-9.

Kishimoto, M., Y. Yano, S. Yajima, S. Otani, T. Ichikawa & T. Yano: The inhibitory effect of vitamin E on 4-(methylnitrosamino)-1-(3- pyridyl)-1-butanone-induced lung tumorigenesis in mice based on the regulation of polyamine metabolism. *Cancer Lett* 1998, **126,** 173-8.

Kitamura, T., S. Watanabe & N. Sato: Liver regeneration, liver cancers and cyclins. *J Gastroenterol Hepatol* 1998, **13 Suppl,** S96-9.

Klaunig, J. E. & L. M. Kamendulis: Mechanisms of cancer chemoprevention in hepatic carcinogenesis: modulation of focal lesion growth in mice. *Toxicol Sci* 1999, **52,** 101-6.

Klaunig, J. E., Y. Xu, S. Bachowski, C. A. Ketcham, J. S. Isenberg, K. L. Kolaja, T. K. Baker, E. F. Walborg, Jr. & D. E. Stevenson: Oxidative stress in nongenotoxic carcinogenesis. *Toxicol Lett* 1995, **82-83,** 683-91.

Klinge, C. M., J. L. Bowers, P. C. Kulakosky, K. K. Kamboj & H. I. Swanson: The aryl hydrocarbon receptor (AHR)/AHR nuclear translocator (ARNT) heterodimer interacts with naturally occurring estrogen response elements. *Mol Cell Endocrinol* 1999, **157,** 105-19.

Kobusch, A. B., G. Fischer & K. W. Bock: Tumor-promoting activity and cytotoxicity of 3,4,3',4'- tetrachlorobiphenyl on N-nitrosomorpholine-induced murine liver foci. *J Cancer Res Clin Oncol* 1989, **115,** 247-52.

Kolaja, K. L., D. T. Engelken & C. D. Klaassen: Inhibition of gap-junctional-intercellular communication in intact rat liver by nongenotoxic hepatocarcinogens. *Toxicology* 2000, **146,** 15-22.

Kolaja, K. L. & J. E. Klaunig: Vitamin E modulation of hepatic focal lesion growth in mice. *Toxicol Appl Pharmacol* 1997, **143,** 380-7.

Kolaja, K. L., D. E. Stevenson, E. F. J. Walborg & J. E. Klaunig: Reversibility of promoter induced hepatic focal lesion growth in mice. *Carcinogenesis* 1996, **17,** 1403-9.

Kolaja, K. L., Y. Xu, E. F. J. Walborg, D. E. Stevenson & J. E. Klaunig: Vitamin E modulation of dieldrin-induced hepatic focal lesion growth in mice. *J Toxicol Environ Health A* 1998, **53,** 479-92.

Kono, H., N. Enomoto, H. D. Connor, M. D. Wheeler, B. U. Bradford, C. A. Rivera, M. B. Kadiiska, R. P. Mason & R. G. Thurman: Medium-chain triglycerides inhibit free radical formation and TNF-alpha production in rats given enteral ethanol. *Am J Physiol Gastrointest Liver Physiol* 2000, **278,** G467-76.

Koremura, N., M. Saito, T. Hasegawa & S. Innami: Effect of polychlorinated biphenyls on tissue distribution and intestinal absorption of alpha-tocopherol in rats. *J Nutr Sci Vitaminol (Tokyo)* 1990, **36,** 457-65.

Kramer, V. J., W. G. Helferich, A. Bergman, E. Klasson-Wehler & J. P. Giesy: Hydroxylated polychlorinated biphenyl metabolites are anti-estrogenic in a stably transfected human breast adenocarcinoma (MCF7) cell line. *Toxicol Appl Pharmacol* 1997, **144,** 363-76.

Krishnan, V. & S. Safe: Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), and dibenzofurans (PCDFs) as antiestrogens in MCF-7 human breast cancer cells: quantitative structure-activity relationships. *Toxicol Appl Pharmacol* 1993, **120,** 55-61.

Laden, F., G. Collman, K. Iwamoto, A. J. Alberg, G. S. Berkowitz, J. L. Freudenheim, S. E. Hankinson, K. J. Helzlsouer, T. R. Holford, H. Y. Huang, K. B. Moysich, J. D. Tessari, M. S. Wolff, T. Zheng & D. J. Hunter: 1,1-Dichloro-2,2-bis(p-chlorophenyl)ethylene and polychlorinated biphenyls and breast cancer: combined analysis of five U.S. studies. *J Natl Cancer Inst* 2001a, **93,** 768-76.

Laden, F., S. E. Hankinson, M. S. Wolff, G. A. Colditz, W. C. Willett, F. E. Speizer & D. J. Hunter: Plasma organochlorine levels and the risk of breast cancer: an extended follow-up in the Nurses' Health Study. *Int J Cancer* 2001b, **91,** 568-74.

Lavoie, J. N., G. L'Allemain, A. Brunet, R. Muller & J. Pouyssegur: Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. *J Biol Chem* 1996, **271,** 20608-16.

Lawrence, J. W., G. K. Wollenberg & J. G. DeLuca: Tumor necrosis factor alpha is not required for WY14,643-induced cell proliferation. *Carcinogenesis* 2001, **22,** 381-6.

Lee, H., H. Dadgostar, Q. Cheng, J. Shu & G. Cheng: NF-kappaB-mediated up-regulation of Bcl-x and Bfl-1/A1 is required for CD40 survival signaling in B lymphocytes. *Proc Natl Acad Sci U S A* 1999, **96,** 9136-41.

Li, N. & M. Karin: Is NF-kappaB the sensor of oxidative stress? *Faseb J* 1999, **13,** 1137-43.

Li, Y., H. P. Glauert & B. T. Spear: Activation of nuclear factor-kappaB by the peroxisome proliferator ciprofibrate in H4IIEC3 rat hepatoma cells and its inhibition by the antioxidants N-acetylcysteine and vitamin E. *: Biochem Pharmacol* 2000, **59,** 427-34.

Li, Y., L. K. Leung, S. B.T. & H. P. Glauert: Activation of hepatic NF-kappaB by phenobarbital in rats. *Biochem Biophys Res Commun* 1996a, **229,** 982-9.

Li, Y., L. K. Leung, H. P. Glauert & B. T. Spear: Treatment of rats with the peroxisome proliferator ciprofibrate results in increased liver NF-kappaB activity. *Carcinogenesis* 1996b, **17,** 2305-9.

Li, Z. W., W. Chu, Y. Hu, M. Delhase, T. Deerinck, M. Ellisman, R. Johnson & M. Karin: The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. *J Exp Med* 1999, **189,** 1839-45.

Lii, C. K., C. W. Chen, J. Y. Liu, Y. J. Ko & H. W. Chen: Lack of effect of dietary alpha-tocopherol on chemically induced hepatocarcinogenesis in rats. *Nutr Cancer* 1999, **34,** 192-8.

Lii, C. K., J. J. Ko & H. W. Chen: No inhibition of gamma-glutamyl transpeptidase-positive foci by vitamin E with or without phenobarbital. *Nutr Cancer* 1997, **27,** 200-5.

Liljegren, G., L. Hardell, G. Lindstrom, P. Dahl & A. Magnuson: Case-control study on breast cancer and adipose tissue concentrations of congener specific polychlorinated biphenyls, DDE and hexachlorobenzene. *Eur J Cancer Prev* 1998, **7,** 135-40.

Liu, H., C. R. Lo & M. J. Czaja: NF-kappaB inhibition sensitizes hepatocytes to TNF-induced apoptosis through a sustained activation of JNK and c-Jun. *Hepatology* 2002, **35,** 772-8. t&artType=abs&id=ajhep0350772&target=.

Loomis, D., S. R. Browning, A. P. Schenck, E. Gregory & D. A. Savitz: Cancer mortality among electric utility workers exposed to polychlorinated biphenyls. *Occup Environ Med* 1997, **54,** 720-8.

Lucena, R. A., M. F. Allam, I. H. Costabeber, M. L. Villarejo & R. F. Navajas: Breast cancer risk factors: PCB congeners. *Eur J Cancer Prev* 2001, **10,** 117-9.

Madra, S., J. Styles & A. G. Smith: Perturbation of hepatocyte nuclear populations induced by iron and polychlorinated biphenyls in C57BL/10ScSn mice during carcinogenesis. *Carcinogenesis* 1995, **16,** 719-27.

Makpol, S., N. A. Shamaan, Z. Jarien, A. G. Top, B. A. Khalid & W. Z. Wan Ngah: Different starting times of alpha-tocopherol and gamma-tocotrienol supplementation and tumor marker enzyme activities in the rat chemically induced with cancer. *Gen Pharmacol* 1997, **28,** 589-92.

Mayo, M. W. & A. S. Baldwin: The transcription factor NF-kappaB: control of oncogenesis and cancer therapy resistance. *Biochim Biophys Acta* 2000, **1470,** M55-62.

McKay, L. I. & J. A. Cidlowski: CBP (CREB binding protein) integrates NF-kappaB (nuclear factor-kappaB) and glucocorticoid receptor physical interactions and antagonism. *Mol Endocrinol* 2000, **14,** 1222-34.

McLean, M. R., U. Bauer, A. R. Amaro & L. W. Robertson: Identification of catechol and hydroquinone metabolites of 4- monochlorobiphenyl. *Chem Res Toxicol* 1996, **9,** 158-64.

McLean, M. R., T. P. Twaroski & L. W. Robertson: Redox cycling of 2-(x'-mono, -di, -trichlorophenyl)- 1, 4- benzoquinones, oxidation products of polychlorinated biphenyls. *Arch Biochem Biophys* 2000, **376,** 449-55.

McVean, M. & D. C. Liebler: Prevention of DNA photodamage by vitamin E compounds and sunscreens: roles of ultraviolet absorbance and cellular uptake. *Mol Carcinog* 1999, **24,** 169-76.

Moore, M. A., H. Tsuda, W. Thamavit, T. Masui & N. Ito: Differential modification of development of preneoplastic lesions in the Syrian golden hamster initiated with a single dose of 2,2'-dioxo-N- nitrosodipropylamine: influence of subsequent butylated hydroxyanisole, alpha-tocopherol, or carbazole. *J Natl Cancer Inst* 1987, **78,** 289-93.

Moysich, K. B., C. B. Ambrosone, J. E. Vena, P. G. Shields, P. Mendola, P. Kostyniak, H. Greizerstein, S. Graham, J. R. Marshall, E. F. Schisterman & J. L. Freudenheim: Environmental organochlorine exposure and postmenopausal breast cancer risk. *Cancer Epidemiol Biomarkers Prev* 1998, **7,** 181-8.

Moysich, K. B., P. G. Shields, J. L. Freudenheim, E. F. Schisterman, J. E. Vena, P. Kostyniak, H. Greizerstein, J. R. Marshall, S. Graham & C. B. Ambrosone: Polychlorinated biphenyls, cytochrome P4501A1 polymorphism, and postmenopausal breast cancer risk. *Cancer Epidemiol Biomarkers Prev* 1999, **8,** 41-4.

Muangmoonchai, R., D. Smirlis, S. C. Wong, M. Edwards, I. R. Phillips & E. A. Shephard: Xenobiotic induction of cytochrome P450 2B1 (CYP2B1) is mediated by the orphan nuclear receptor constitutive androstane receptor (CAR) and requires steroid co-activator 1 (SRC-1) and the transcription factor Sp1. *Biochem J* 2001, **355,** 71-8.

Nakamura, T., M. Goto, A. Matsumoto & I. Tanaka: Inhibition of NF-kappa B transcriptional activity by alpha-tocopheryl succinate. *Biofactors* 1998, **7,** 21-30.

Nakanishi, Y., F. Bai, K. Inoue, K. Takayama, X. H. Pei, T. Harada, M. Izumi, K. Kimotsuki, H. Tokiwa & N. Hara: Polychlorinated biphenyls promote 1-nitropyrene-induced lung tumorigenesis without the induction of K-ras gene mutation in A/J mice. *Teratog Carcinog Mutagen* 2001, **21,** 395-403.

Nakanishi, Y., N. Shigematsu, Y. Kurita, K. Matsuba, H. Kanegae, S. Ishimaru & Y. Kawazoe: Respiratory involvement and immune status in yusho patients. *Environ Health Perspect* 1985, **59,** 31-6.

Nesaretnam, K. & P. Darbre: 3,5,3',5'-tetrachlorobiphenyl is a weak oestrogen agonist in vitro and in vivo. *J Steroid Biochem Mol Biol* 1997, **62,** 409-18.

Nesaretnam, K., E. Hales, M. Sohail, T. Krausz & P. Darbre: 3,3',4,4'-tetrachlorobiphenyl (TCB) can enhance DMBA-induced mammary carcinogenesis in the rat. *Eur J Cancer* 1998, **34,** 389-93.

Nilakantan, V., B. T. Spear & H. P. Glauert: Liver-specific catalase expression in transgenic mice inhibits NF-kappaB activation and DNA synthesis induced by the peroxisome proliferator ciprofibrate. *Carcinogenesis* 1998, **19,** 631-7.

Nishizumi, M.: Enhancement of diethylnitrosamine hepatocarcinogenesis in rats by exposure to polychlorinated biphenyls or phenobarbital. *Cancer Lett* 1976, **2,** 11-5.

Nissen, R. M. & K. R. Yamamoto: The glucocorticoid receptor inhibits NFkappaB by interfering with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain. *Genes Dev* 2000, **14,** 2314-29.

Nyandieka, H. S., J. Wakhis & M. M. Kilonzo: Association of reduction of AFB1-induced liver tumours by antioxidants with increased activity of microsomal enzymes. *Indian J Med Res* 1990, **92,** 332-6.

Nyandieka, H. S. & J. Wakhisi: The impact of vitamins A,C,E, and selenium compound on prevention of liver cancer in rats. *East Afr Med J* 1993, **70,** 151-3.

Nyandieka, H. S., J. Wakhisi & M. Kilonzo: Inhibition of AFB1-induced liver cancer and induction of increased microsomal enzyme activity by dietary constituents. *East Afr Med J* 1989, **66,** 796-803.

Oakley, G. G., U. Devanaboyina, L. W. Robertson & R. C. Gupta: Oxidative DNA damage induced by activation of polychlorinated biphenyls (PCBs): implications for PCB-induced oxidative stress in breast cancer. *Chem Res Toxicol* 1996, **9,** 1285-92.

Oakley, G. G., A. L. Roe, R. A. Blouin, T. P. Twaroski, T. C. Ganguly, M. Vore, H. J. Lehmler & L. W. Robertson: 2,4,4'-trichlorobiphenyl increases STAT5 transcriptional activity. *Mol Carcinog* 2001, **30,** 199-208.

Odeleye, O. E., C. D. Eskelson, S. I. Mufti & R. R. Watson: Vitamin E inhibition of lipid peroxidation and ethanol-mediated promotion of esophageal tumorigenesis. *Nutr Cancer* 1992, **17,** 223-34.

Oesterle, D. & E. Deml: Promoting effect of polychlorinated biphenyls on development of enzyme- altered islands in livers of weanling and adult rats. *J Cancer Res Clin Oncol* 1983, **105,** 141-7.

Ogawa, T., S. Higashi, Y. Kawarada & R. Mizumoto: Role of reactive oxygen in synthetic estrogen induction of hepatocellular carcinomas in rats and preventive effect of vitamins. *Carcinogenesis* 1995, **16,** 831-6.

Ohshima, H., J. C. Bereziat & H. Bartsch: Monitoring N-nitrosamino acids excreted in the urine and feces of rats as an index for endogenous nitrosation. *Carcinogenesis* 1982, **3,** 115-20.

Okumura, M.: Past and current medical states of yusho patients. *Prog Clin Biol Res* 1984, **137,** 13-8.

Parkinson, A., S. H. Safe, L. W. Robertson, P. E. Thomas, D. E. Ryan, L. M. Reik & W. Levin: Immunochemical quantitation of cytochrome P-450 isozymes and epoxide hydrolase in liver microsomes from polychlorinated or polybrominated biphenyl-treated rats. A study of structure-activity relationships. *J Biol Chem* 1983, **258,** 5967-76.

Pelissier, M. A., M. Boisset, S. Atteba & R. Albrecht: Lipid peroxidation of rat liver microsomes membranes related to a protein deficiency and/or a PCB treatment. *Food Addit Contam* 1990, **7,** S172-7.

Pelissier, M. A., M. H. Siess, M. Lhuissier, P. Grolier, M. Suschetet, J. F. Narbonne, R. Albrecht & L. W. Robertson: Effect of prototypic polychlorinated biphenyls on hepatic and renal vitamin contents and on drug-metabolizing enzymes in rats fed diets containing low or high levels of retinyl palmitate. *Food Chem Toxicol* 1992, **30,** 723-9.

Peltola, V., E. Mantyla, I. Huhtaniemi & M. Ahotupa: Lipid peroxidation and antioxidant enzyme activities in the rat testis after cigarette smoke inhalation or administration of polychlorinated biphenyls or polychlorinated naphthalenes. *J Androl* 1994, **15,** 353-61.

Pereg, D., L. W. Robertson & R. C. Gupta: DNA adduction by polychlorinated biphenyls: adducts derived from hepatic microsomal activation and from synthetic metabolites. *Chem Biol Interact* 2002, **139,** 129-44.

Pereira, M. A., S. L. Herren, A. L. Britt & M. M. Khoury: Promotion by polychlorinated biphenyls of enzyme-altered foci in rat liver. *Cancer Lett* 1982, **15,** 185-90.

Pitot, H. C. & Y. P. Dragan: The multistage nature of chemically induced hepatocarcinogenesis in the rat. *Drug Metab Rev* 1994, **26,** 209-20.

Preston, B. D., E. C. Miller & J. A. Miller: The activities of 2,2',5,5'-tetrachlorobiphenyl, its 3,4-oxide metabolite, and 2,2',4,4'-tetrachlorobiphenyl in tumor induction and promotion assays. *Carcinogenesis* 1985, **6,** 451-3.

Preston, B. D., J. P. Van Miller, R. W. Moore & J. R. Allen: Promoting effects of polychlorinated biphenyls (Aroclor 1254) and polychlorinated dibenzofuran-free

Aroclor 1254 on diethylnitrosamine- induced tumorigenesis in the rat. *J Natl Cancer Inst* 1981, **66,** 509-15.

Puga, A., S. J. Barnes, C. Chang, H. Zhu, K. P. Nephew, S. A. Khan & H. G. Shertzer: Activation of transcription factors activator protein-1 and nuclear factor-kappaB by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Biochem Pharmacol* 2000, **59,** 997-1005.

Ramakrishna, G. & L. M. Anderson: Levels and membrane localization of the c-K-ras p21 protein in lungs of mice of different genetic strains and effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and Aroclor 1254. *Carcinogenesis* 1998, **19,** 463-70.

Ramamoorthy, K., M. S. Gupta, G. Sun, A. McDougal & S. H. Safe: 3,3'4,4'-Tetrachlorobiphenyl exhibits antiestrogenic and antitumorigenic activity in the rodent uterus and mammary cells and in human breast cancer cells. *Carcinogenesis* 1999, **20,** 115-23.

Ramamoorthy, K., C. Vyhlidal, F. Wang, I. Chen, S. Safe, D. P. McDonnell, L. S. Leonard & K. W. Gaido: Additive estrogenic activities of a binary mixture of 2',4',6'- trichloro- and 2',3',4',5'-tetrachloro-4-biphenylol. *Toxicol Appl Pharmacol* 1997, **147,** 93-100.

Roberts, R. A., A. R. Soames, J. H. Gill, N. H. James & E. B. Wheeldon: Non-genotoxic hepatocarcinogens stimulate DNA synthesis and their withdrawal induces apoptosis, but in different hepatocyte populations. *Carcinogenesis* 1995, **16,** 1693-8.

Rose, M. L., D. Germolec, G. E. Arteel, R. Schoonhoven & R. G. Thurman: Dietary glycine prevents increases in hepatocyte proliferation caused by the peroxisome proliferator WY-14,643. *Chem Res Toxicol* 1997a, **10,** 1198-204.

Rose, M. L., D. R. Germolec, R. Schoonhoven & R. G. Thurman: Kupffer cells are causally responsible for the mitogenic effect of peroxisome proliferators. *Carcinogenesis* 1997b, **18,** 1453-6.

Rose, M. L., I. Rusyn, H. K. Bojes, J. Belyea, R. C. Cattley & R. G. Thurman: Role of Kupffer cells and oxidants in signaling peroxisome proliferator- induced hepatocyte proliferation. *Mutat Res* 2000, **448,** 179-92.

bin/cas/tree/store/mut/cas_sub/browse/browse.cgi?year=2000&volume=448&issue=2&aid=8905.

Rosinska, A. & W. Sulkowski: Selected PCB Congener Determination by Means of CGC-MS and CGC-ECD in Water Extracts. *PCBs: Recent Advances in Environmental Toxicology and Health Effects* 2001**,** 77-80.

Rudolph, D., W. C. Yeh, A. Wakeham, B. Rudolph, D. Nallainathan, J. Potter, A. J. Elia & T. W. Mak: Severe liver degeneration and lack of NF-kappaB activation in NEMO/IKKgamma-deficient mice. *Genes Dev* 2000, **14,** 854-62.

Runge-Morris, M.: Regulation of sulfotransferase gene expression by glucocorticoid hormones and xenobiotics in primary rat hepatocyte culture. *Chem Biol Interact* 1998, **109,** 315-27.

Rusyn, I., C. A. Bradham, L. Cohn, R. Schoonhoven, J. A. Swenberg, D. A. Brenner & R. G. Thurman: Corn oil rapidly activates nuclear factor-kappaB in hepatic Kupffer cells by oxidant-dependent mechanisms. *Carcinogenesis* 1999, **20,** 2095-100.

Safe, S., S. Bandiera, T. Sawyer, L. Robertson, L. Safe, A. Parkinson, P. E. Thomas, D. E. Ryan, L. M. Reik, W. Levin & et al.: PCBs: structure-function relationships and mechanism of action. *Environ Health Perspect* 1985, **60,** 47-56.

Safe, S. H.: Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. *Crit Rev Toxicol* 1994, **24,** 87-149.

Safe, S. H.: Is there an association between exposure to environmental estrogens and breast cancer? *Environ Health Perspect* 1997, **105 Suppl 3,** 675-8.

Saito, M.: Polychlorinated biphenyls-induced lipid peroxidation as measured by thiobarbituric acid-reactive substances in liver subcellular fractions of rats. *Biochim Biophys Acta* 1990, **1046,** 301-8.

Salvi, M. & A. Toninello: Aroclor 1254 inhibits the mitochondrial permeability transition and release of cytochrome c: a possible mechanism for its in vivo toxicity. *Toxicol Appl Pharmacol* 2001, **176,** 92-100.

Sanders, S. & S. S. Thorgeirsson: Phenobarbital promotes liver growth in c-myc/TGF-alpha transgenic mice by inducing hypertrophy and inhibiting apoptosis. *Carcinogenesis* 1999, **20,** 41-9.

Sanders, S. & S. S. Thorgeirsson: Promotion of hepatocarcinogenesis by phenobarbital in c-myc/TGF-alpha transgenic mice. *Mol Carcinog* 2000, **28,** 168-73.

Sargent, L., Y. P. Dragan, C. Erickson, C. J. Laufer & H. C. Pitot: Study of the separate and combined effects of the non-planar 2,5,2',5'- and the planar 3,4,3',4'-tetrachlorobiphenyl in liver and lymphocytes in vivo. *Carcinogenesis* 1991, **12,** 793-800.

Scheinman, R. I., P. C. Cogswell, A. K. Lofquist & A. S. Baldwin, Jr.: Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. *Science* 1995, **270,** 283-6.

Schramm, H., L. W. Robertson & F. Oesch: Differential regulation of hepatic glutathione transferase and glutathione peroxidase activities in the rat. *Biochem Pharmacol* 1985, **34,** 3735-9.

Schreck, R., K. Albermann & P. A. Baeuerle: Nuclear factor kappa B: an oxidative stress-responsive transcription factor of eukaryotic cells (a review). *Free Radic Res Commun* 1992, **17,** 221-37.

Schuetz, E. G., C. Brimer & J. D. Schuetz: Environmental xenobiotics and the antihormones cyproterone acetate and spironolactone use the nuclear hormone pregnenolone X receptor to activate the CYP3A23 hormone response element. *Mol Pharmacol* 1998, **54,** 1113-7.

Schwabe, R. F. & D. A. Brenner: Role of glycogen synthase kinase-3 in TNF-alpha-induced NF-kappaB activation and apoptosis in hepatocytes. *Am J Physiol Gastrointest Liver Physiol* 2002, **283,** G204-11.

Sha, W., H. Liou, E. Tuomanen & D. Baltimore: Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. *Cell* 1995, **80,** 321-30.

Shane, B. S., D. L. Smith-Dunn, J. G. deBoer, B. W. Glickman & M. L. Cunningham: Subchronic administration of phenobarbital alters the mutation spectrum of lacI in the livers of Big Blue transgenic mice. *Mutat Res* 2000, **448,** 69-80.

Shekhar, P. V., J. Werdell & V. S. Basrur: Environmental estrogen stimulation of growth and estrogen receptor function in preneoplastic and cancerous human breast cell lines. *J Natl Cancer Inst* 1997, **89,** 1774-82.

Sheppard, K. A., K. M. Phelps, A. J. Williams, D. Thanos, C. K. Glass, M. G. Rosenfeld, M. E. Gerritsen & T. Collins: Nuclear integration of glucocorticoid receptor and nuclear factor- kappaB signaling by CREB-binding protein and steroid receptor coactivator-1. *J Biol Chem* 1998, **273,** 29291-4.

Silberhorn, E. M., H. P. Glauert & L. W. Robertson: Carcinogenicity of polyhalogenated biphenyls: PCBs and PBBs. *Crit Rev Toxicol* 1990, **20,** 440-96.

Silkworth, J. B. & J. F. Brown, Jr.: Evaluating the impact of exposure to environmental contaminants on human health. *Clin Chem* 1996, **42,** 1345-9.

Sinks, T., G. Steele, A. B. Smith, K. Watkins & R. A. Shults: Mortality among workers exposed to polychlorinated biphenyls. *Am J Epidemiol* 1992, **136,** 389-98.

Slim, R., M. Toborek, L. W. Robertson & B. Hennig: Antioxidant protection against PCB-mediated endothelial cell activation. *Toxicol Sci* 1999, **52,** 232-9.

Slim, R., M. Toborek, L. W. Robertson, H. J. Lehmler & B. Hennig: Cellular glutathione status modulates polychlorinated biphenyl-induced stress response and apoptosis in vascular endothelial cells. *Toxicol Appl Pharmacol* 2000, **166,** 36-42.

Sovak, M. A., R. E. Bellas, D. W. Kim, G. J. Zanieski, A. E. Rogers, A. M. Traish, and G. E. Sonenshein.: Aberrant nuclear factor-kappaB/Rel expression and the pathogenesis of breast cancer. *J. Clin. Investig.* 1997, **100,** 2952-60.

Srinivasan, A., H. J. Lehmler, L. W. Robertson & G. Ludewig: Production of DNA strand breaks in vitro and reactive oxygen species in vitro and in HL-60 cells by PCB metabolites. *Toxicol Sci* 2001, **60,** 92-102.

Steer, J. H., K. M. Kroeger, L. J. Abraham & D. A. Joyce: Glucocorticoids suppress tumor necrosis factor-alpha expression by human monocytic THP-1 cells by suppressing transactivation through adjacent NF-kappa B and c-Jun-activating transcription factor-2 binding sites in the promoter. *J Biol Chem* 2000, **275,** 18432-40.

Stevenson, D. E., J. P. Kehrer, K. L. Kolaja, E. F. Walborg, Jr. & J. E. Klaunig: Effect of dietary antioxidants on dieldrin-induced hepatotoxicity in mice. *Toxicol Lett* 1995, **75,** 177-83.

Tai, D. I., S. L. Tsai, Y. H. Chang, S. N. Huang, T. C. Chen, K. S. Chang & Y. F. Liaw: Constitutive activation of nuclear factor kappaB in hepatocellular carcinoma. *Cancer* 2000, **89,** 2274-81.

Tamatani, M., Y. Che, H. Matsuzaki, S. Ogawa, H. Okado, S. Miyake, T. Mizuno & M. Tohyama: Tumor necrosis factor induces Bcl-2 and Bcl-x expression through NFkappaB activation in primary hippocampal neurons. *J Biol Chem* 1999, **274,** 8531-8.

Tanaka, M., M. E. Fuentes, K. Yamaguchi, M. H. Durnin, S. A. Dalrymple, K. L. Hardy & D. V. Goeddel: Embryonic lethality, liver degeneration, and impaired NF-kappa B activation in IKK-beta-deficient mice. *Immunity* 1999, **10,** 421-9.

Tang, G., Y. Minemoto, B. Dibling, N. H. Purcell, Z. Li, M. Karin & A. Lin: Inhibition of JNK activation through NF-kappaB target genes. *Nature* 2001, **414,** 313-7.

Tatematsu, M., K. Nakanishi, G. Murasaki, Y. Miyata, M. Hirose & N. Ito: Enhancing effect of inducers of liver microsomal enzymes on induction of hyperplastic liver nodules by N-2-fluorenylacetamide in rats. *J Natl Cancer Inst* 1979, **63,** 1411-6.

Tharappel, J. C., Spear, B.T., Glauert, H.P.: The Effect of the Peroxisome Proliferator Ciprofibrate on Cell Proliferation and Apoptosis in Mice Deficient in the p50 subunit of NF-κB. *Toxicological Sciences* 2001, **60,** 757.

Tharappel, J. C., E. Y. Lee, L. W. Robertson, B. T. Spear & H. P. Glauert: Regulation of Cell Proliferation, Apoptosis, and Transcription Factor Activities during the Promotion of Liver Carcinogenesis by Polychlorinated Biphenyls. *Toxicol Appl Pharmacol* 2002, **179,** 172-184.

Tian, Y., S. Ke, M. S. Denison, A. B. Rabson & M. A. Gallo: Ah receptor and NF-kappaB interactions, a potential mechanism for dioxin toxicity. *J Biol Chem* 1999, **274,** 510-5.

Twaroski, T. P., M. L. O'Brien, N. Larmonier, H. P. Glauert & L. W. Robertson: Polychlorinated biphenyl-induced effects on metabolic enzymes, AP-1 binding,

vitamin E, and oxidative stress in the rat liver. *Toxicol Appl Pharmacol* 2001a, **171,** 85-93.

Twaroski, T. P., M. L. O'Brien & L. W. Robertson: Effects of selected polychlorinated biphenyl (PCB) congeners on hepatic glutathione, glutathione-related enzymes, and selenium status: implications for oxidative stress. *Biochem Pharmacol* 2001b, **62,** 273-81.

Van Ess, P. J., M. P. Mattson & R. A. Blouin: Enhanced induction of cytochrome P450 enzymes and CAR binding in TNF (p55(-/-)/p75(-/-)) double receptor knockout mice following phenobarbital treatment. *J Pharmacol Exp Ther* 2002, **300,** 824-30.

van Poppel, G. & H. van den Berg: Vitamins and cancer. *Cancer Lett* 1997, **114,** 195-202.

Wang, C. Y., D. C. Guttridge, M. W. Mayo & A. S. J. Baldwin: NF-kappaB induces expression of the Bcl-2 homologue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis. *Mol Cell Biol* 1999, **19,** 5923-9.

Wang, C. Y., M. W. Mayo, R. G. Korneluk, D. V. Goeddel & A. S. Baldwin, Jr.: NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c- IAP2 to suppress caspase-8 activation. *Science* 1998, **281,** 1680-3.

Wang, T., R. Cardiff, L. Zukerberg, E. Lees, A. Arnold & E. Schmidt: Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. *Nature* 1994, **369,** 669-71.

Ward, E. M., P. Schulte, B. Grajewski, A. Andersen, D. G. Patterson, Jr., W. Turner, E. Jellum, J. A. Deddens, J. Friedland, N. Roeleveld, M. Waters, M. A. Butler, E. DiPietro & L. L. Needham: Serum organochlorine levels and breast cancer: a nested case-control study of Norwegian women. *Cancer Epidemiol Biomarkers Prev* 2000, **9,** 1357-67.

Watanabe, T., R. Pakala, T. Katagiri & C. R. Benedict: Lipid peroxidation product 4-hydroxy-2-nonenal acts synergistically with serotonin in inducing vascular smooth muscle cell proliferation. *Atherosclerosis* 2001, **155,** 37-44.

Welsch, C. W.: Review of the effects of dietary fat on experimental mammary gland tumorigenesis: role of lipid peroxidation. *Free Radic Biol Med* 1995, **18,** 757-73.

Whysner, J. & C. X. Wang: Hepatocellular iron accumulation and increased cell proliferation in polychlorinated biphenyl-exposed sprague-dawley rats and the development of hepatocarcinogenesis. *Toxicol Sci* 2001, **62,** 36-45.

Wolff, M. S., P. G. Toniolo, E. W. Lee, M. Rivera & N. Dubin: Blood levels of organochlorine residues and risk of breast cancer. *J Natl Cancer Inst* 1993, **85,** 648-52.

Wolff, M. S., A. Zeleniuch-Jacquotte, N. Dubin & P. Toniolo: Risk of breast cancer and organochlorine exposure. *Cancer Epidemiol Biomarkers Prev* 2000, **9,** 271-7.

Wolfle, D., P. Munzel, G. Fischer & K. W. Bock: Altered growth control of rat hepatocytes after treatment with 3,4,3',4'-tetrachlorobiphenyl in vivo and in vitro. *Carcinogenesis* 1988, **9,** 919-24.

Xu, Y. H., Y. P. Dragan, H. A. Campbell & H. C. Pitot: STEREO: a program on a PC-Windows 95 platform for recording and evaluating quantitative stereologic investigations of multistage hepatocarcinogenesis in rodents. *Comput Methods Programs Biomed* 1998, **56,** 49-63.

Yamamoto, K., N. Fukuda, S. Shiroi, Y. Shiotsuki, Y. Nagata, T. Tani & T. Sakai: Ameliorative effect of dietary probucol on polychlorinated biphenyls- induced hypercholesterolemia and lipid peroxidation in the rat. *Life Sci* 1994, **54,** 1019-26.

Yamashita, F. & M. Hayashi: Fetal PCB syndrome: clinical features, intrauterine growth retardation and possible alteration in calcium metabolism. *Environ Health Perspect* 1985, **59,** 41-5.

Yassi, A., R. Tate & D. Fish: Cancer mortality in workers employed at a transformer manufacturing plant. *Am J Ind Med* 1994, **25,** 425-37.

Yu, M. L., Y. L. Guo, C. C. Hsu & W. J. Rogan: Increased mortality from chronic liver disease and cirrhosis 13 years after the Taiwan "yucheng" ("oil disease") incident. *Am J Ind Med* 1997, **31,** 172-5.

Zheng, T., T. R. Holford, S. T. Mayne, J. Tessari, B. Ward, D. Carter, P. H. Owens, P. Boyle, R. Dubrow, S. Archibeque-Engle, O. Dawood & S. H. Zahm: Risk of female breast cancer associated with serum polychlorinated biphenyls and 1,1-dichloro-2,2'-bis(p-chlorophenyl)ethylene. *Cancer Epidemiol Biomarkers Prev* 2000a, **9,** 167-74.

Zheng, T., T. R. Holford, J. Tessari, S. T. Mayne, P. H. Owens, B. Ward, D. Carter, P. Boyle, R. Dubrow, S. Archibeque-Engle & S. H. Zahm: Breast cancer risk associated with congeners of polychlorinated biphenyls. *Am J Epidemiol* 2000b, **152,** 50-8.

Vita

Name: Zijing Lu

Date of Birth: November 12, 1972

Place of Birth: Heilongjinag, China

Education:

M.S. Biochemistry, Fudan University (1993-1996)

Shanghai, China

B.S. Biochemistry, Fudan University (1989-1993)

Shanghai, China

Professional positions held:

Research Assistant, Nutritional Sciences, University of Kentucky (1997-2002)

Research and Development Specialist, Unilever Shanghai, Co. Ltd. (1996-1997)

Shanghai, China

Research Assiatant, Department of Biochemistry, Fudan University (1993-1996)

Shanghai, China

Scholastic and professional honors:

Third place in Student Awards Competition, the Chemical Carcinogenesis Specialty Section of the Society of Toxicology (2002)

Recipient of a Kentucky Opportunity Fellowship, University of Kentucky (2001-2002)

Recipient of a Dissertation Enhancement Award, University of Kentucky (2001)

Recipient of the Training Core of the Superfund Basic Research Program, NIEHS (2000-2001)

Publications:

Chengbin Lin, Stephen G. Zimmer, **Zijing Lu**, Robert E. Holland Jr., Qing Dong, and Thomas M. Chambers. The Involvement of a Stress-Activated Pathway in Equine Influenza Virus-Mediated Apoptosis. *Virology* 287, 202-213 (2001)

Zijing Lu, Brett Spear, Eun Y. Lee, Larry Robertson, and Howard P. Glauert. Activation of Rat Hepatic Transcription Factor NF-κB By PCBs. *PCBs: Recent Advances in the Environmental Toxicology and Health Effects.* 419-420, The University Press of Kentucky (2001).

Zaineb Fadhel, **Zijing Lu**, Larry W. Robertson, and Howard P. Glauert. Effect of 3,3',4,4'-Tetrachlorobiphenyl and 2,2',4,4',5,5'-Hexachlorobiphenyl on the Induction of Hepatic Lipid Peroxidation and Cytochrome P-450 Associated Enzyme Activities in Rats. *Toxicology* 175, 15-25 (2002)

Zijing Lu, Brett Spear, Eun Y. Lee, Larry Robertson, and Howard P. Glauert. Increased Hepatic NF-κB DNA Binding Activity and Cell proliferation after a Single Dose of Polychlorinated Biphenyls in Rats. (Submitted)

Zijing Lu, E. Y. Lee, L. W. Robertson, B. T. Spear and H. P. Glauert. Effect of Dietary Vitamin E on Cell Proliferation and Transcription Factor NF-κB Activity during the Promotion of Liver Carcinogenesis by Polychlorinated Biphenyls. (Manuscript in preparation)

Zijing Lu, E. Y. Lee, L. W. Robertson, B. T. Spear and H. P. Glauert. Effect of 2,2',4,4',5,5'-Haxachlorobiphenyl (PCB-153) on Hepatocyte Proliferation and Apoptosis in Mice Deficient in the p50 Subunit of NF-κB. (Manuscript in preparation)

Abstracts

Glauert, H.P., Y. Li, V. Nilakantan, J.C. Tharappel, K.G. Calfee, **Z. Lu**, L.W. Robertson, and B.T. Spear. Role of NF-κB in the promotion of hepatocarcinogenesis. XIIIth Congress of the Polish Pharmacological Society, Katowice, Poland, Sept. 13-16, 1998. Polish J. Pharmacol. 50 (supplement):80-81.

Z. Lu, B. T. Spear, E. Lee, L. W. Robertson and H. P. Glauert. Activation of Hepatic NF-κB by a Single Dose of Polychlorinated Biphenyls (PCBs). *The FASEB Journal* 13(5), 681.10, 1999.

Z. Lu, B. T. Spear, L. W. Robertson and H. P. Glauert. Activation of Hepatic NF-κB by Polychlorinated Biphenyls (PCBs) *in vivo* and in Cultured Rat Hepatocytes. *Toxicological Sciences* 54 (1), 1192, 2000

Z. A. Fadhel, **Z. Lu**, L. W. Robertson and H. P. Glauert. Role of Cytochrome P450 Induction and Lipid Peroxidation in 3,3',4,4'-Tetrachlorobiphenyl (PCB-77) and 2,2',4,4',5,5'-Hexachlorobiphenyl (PCB-153)-Induced Toxicity in Liver of Male Rats. *Toxicological Sciences* 54 (1), 368, 2000.

Z. Lu, E. Y. Lee, L. W. Robertson, B. T. Spear and H. P. Glauert. Effect of Dietary Vitamin E on the Promotion of Altered Hepatic Foci By 3,3',4,4'-Tetrachlorobiphenyl (PCB-77) and 2,2',4,4',5,5'-Hexachlorobiphenyl (PCB-153) in Rats Initiated with Diethylnitrosamnine. *Toxicological Sciences* 60 (1), 767 (2001).

H.P. Glauert, K. Calfee-Mason, Y. Li, **Z. Lu**, V. Nilakantan, M. O'Brien, J. Tharappel, E.Y. Lee, L.W. Robertson and B.T. Spear. Role of Oxidative Stress in the Promotion of Hepatocarcinogenesis by Environmental Chemicals. 44th Annual Meeting of the Polish

Chemical Society in Katowice, Poland, September 9-13, 2001. To be published in the *Annals of the Polish Chemical Society*.

Z. Lu, E. Y. Lee, L. W. Robertson, B. T. Spear and H. P. Glauert. Effect of 2,2',4,4',5,5'-Haxachlorobiphenyl (PCB-153) on Hepatocyte Proliferation in Mice Deficient in the p50 Subunit of NF-κB. *Toxicological Sciences* 66 (1), 1497 (2002).